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PROCEEDINGS OF THE
AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

SIXTH ANNUAL MEETING

Baltimore and Washington, December 27-29, 1911

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

INTESTINAL PUTREFACTION AND BACTERIAL DEVELOPMENT ACCOMPANYING WATER DRINKING AND FASTING

By N R BLATHERWICK, C P SHERWIN AND P B HAWK

*(From the Laboratory of Physiological Chemistry of the University of
Illinois)*

Data were presented which indicated a marked decrease in the output of bacteria in the feces when normal men were caused to increase their water ingestion by 3450 cc per day, the water being taken with meals. That intestinal putrefaction was also diminished under these conditions was indicated by an accompanying decrease in the urinary indican values during the interval of high water intake. The course of the total ethereal sulphate excretion did not run parallel with that of indican thus furnishing additional evidence in favor of the view that indican has an origin different from that of the other ethereal sulphates.

When a normal man passed into a 7-day fast from a high protein level it was found that the daily output of fecal bacteria was markedly lowered, with a return to normal values with the inception of a post-fasting period of low protein character. Indican and total ethereal sulphates were also decreased under the fasting regime, this decrease being followed by an increase upon the subsequent ingestion of food.

SOME ESSENTIAL CONDITIONS OF ACCURACY AND SPEED FOR THE DETERMINATION OF SUGAR BY THE METHOD OF COP- PER REDUCTION

By AMOS W. PETERS

(From the Carnegie Nutrition Laboratory, Boston, Mass.)

A critical study was made of the conditions which must be observed in the determination of sugar by copper reduction methods when the greatest accuracy and speed are required combined

in one procedure It was found that more exact definition or standardization of known conditions was needed A procedure was developed which is characterized by (a) exact measurement of copper, (b) the standardization of heating power and other conditions of reduction so that perfectly uniform and always reproducible reduction values are obtained, due to a quantitative specification of conditions The heating power is measured by the time (120 seconds) required to raise the temperature of distilled water under specifications through the temperature interval of 35°C to 95°C In a sugar determination a specified form of apparatus is used which is the same as for the measurement of the heating power and is used under the same conditions However, in sugar estimations the only observed time for heating is that of twenty seconds after the thermometer shows a temperature of 95°C A curve was determined showing the amount of reduction in relation to the rise of temperature About 70 per cent of the total reduction has already occurred when the thermometer reaches 75°C The short period of heating is related to this fact and other reducing substances thus have a minimal effect The reduction mixture is filtered hot through a described filtering tube The copper of the filtrate is determined by the iodide method under conditions of accuracy that have been separately studied and reported Limits of error on tabular values and their reproducibility have been tested and the results shown by curves The procedure has also been tested for the quantitative analytical recovery of pure dextrose added to diabetic urine and has yielded the amount added to within a fraction of 1 per cent The procedure has a range of 2 to 175 mgs of dextrose and when the standard conditions have once been established occupies not more than fifteen minutes It is especially reliable for the determination of small reducing powers

ON THE FATE OF INGESTED FAT IN THE ANIMAL BODY

By H S RAPER

(From the Department of Pathological Chemistry, University of Toronto)

Cocoonut oil yielding 40 per cent of acids volatile with steam, can be recovered from a mixture of 1 gram of the oil with the minced liver of a cat to the extent of about 80 per cent, when the fatty acids are subjected to steam distillation If the oil is fed to cats

considerable quantities of volatile acids are obtained from the liver. If an emulsion of the oil be slowly infused into a vein, from 30 to 50 per cent of the volatile acids entering the systemic circulation can be recovered from the liver two or three hours later. About the same proportion of the oil absorbed from the intestine is similarly found in the liver.

THE PURINES AND PURINE ENZYMES OF TUMORS

By H. GIDEON WELLS

(From the Department of Pathology, University of Chicago.)

As the liver is the chief or only organ of the human body capable of oxidizing xanthine into uric acid *in vitro*, the demonstration that secondary carcinomas of the liver are not capable of accomplishing this oxidation is a point in support of the view that the chemical activities of tumor tissue, like their histological structure, breed true in secondary growths and do not correspond to the tissues in which they are growing. Both malignant and benign tumors were found to resemble normal tissues as regards purine content and purine enzymes. Guanase and nucleases seem to be always present, adenase and xanthine oxidase are absent. Carcinomas contain usually somewhat less combined purine nitrogen than such organs as the liver and kidney, averaging about 1.5 per cent of the total fixed nitrogen, fibromyomas contain about 1.1 per cent of purine N. The guanine was usually found to slightly exceed in amount the adenine, and more or less hypoxanthine is always present. A relatively large proportion of hypoxanthine was found in a uterine myoma, corresponding with Saikū's observation that non-striated muscle contains considerable free hypoxanthine.

THE HAEMOLYTIC POWER OF FATTY ACIDS

By FLETCHER MCPHEDRAN

(From the Laboratory of Pathological Chemistry of the University of Toronto.)

Faust and Tallquist ascribed the anaemia of *Bothriocephalus latus* to the unsaturation of oleic acid. Other more unsaturated acids occur in the body and these might be of importance in anaemia. Experiments show that increase in the number of unsaturated carbon atoms does not increase the haemolytic power.

Saturation of the free bonds with halogens seems to actually increase the haemolytic ability, saturation with hydroxyl groups diminishes it in the case of dioxy stearic acid

NOTE ON A NEW SALT OF β -OXYBUTYRIC ACID

By P A SHAFFER

(From the Laboratory of Biological Chemistry, Washington University, St Louis)

Zinc-calcium double salt of the composition $\text{ZnCa}(\text{C}_4\text{H}_7\text{O}_3)_4$ is useful for the purification of β -oxybutyric acid It is prepared by pouring together equivalent parts of the zinc and calcium β -oxy butyrates, made by treating the free acid with zinc and calcium carbonates, respectively The salt is precipitated, crystallized in needles or long narrow plates, on the addition to the warmed solution of an equal volume of alcohol From the purified salt the free acid may be obtained by removal of Zn by H_2S and Ca by the theoretical amount of oxalic acid, or a solution of the salt, acidified with a slight excess of H_2SO_4 , and dehydrated by plaster or anhydrous Na_2SO_4 , may be extracted with dry ether

The salt prepared from 1-oxybutyric acid has a specific rotation of $[\alpha]_D^{25} = -15.1^\circ$ (5 per cent solution)

ON THE ALLANTOIN OUTPUT OF MAN AS INFLUENCED BY WATER INGESTION

By LAWRENCE T FAIRHALL AND P B HAWK

(From the Laboratory of Physiological Chemistry, University of Illinois)

When the normal diet of a normal man was supplemented by 900 cc of water per day the average daily output of allantoin was 0.0135 gram for a period of thirteen days, the determinations being made by Wiechowski's method Upon increasing the water intake to 3450 cc per day for a period of five days, the average daily allantoin excretion was increased to 0.0173 gram The daily value for a five-day final period on the original 900 cc water ingestion was 0.0122 gram

The increase in the allantoin output accompanying water drinking is believed to indicate that the oxidative mechanism of the organism has been stimulated through the introduction of the large

volume of water into the body and that purine material which would ordinarily have been excreted in some less highly oxidized form has been oxidized to allantoin and excreted in this form. This conclusion is substantiated by the findings reported in this laboratory of a decreased output of uric acid accompanying an increased water ingestion by man.

In view of the fact that the above interpretation is contrary to current ideas regarding purine metabolism, the authors make the interpretation *tentatively* until further experiments may be completed.

PHYSIOLOGICAL EFFECTS ON GROWTH AND REPRODUCTION OF RATIONS BALANCED FROM RESTRICTED SOURCES ¹

By E. B. HART, E. V. McCOLLUM AND H. STEENBOCK

(From the Laboratory of Agricultural Chemistry, University of Wisconsin)

This paper summarizes the preliminary results of an extended investigation on the physiological value of rations for domestic animals. Our first experiments were limited to growing and reproducing heifers and extended over a period of four years. There is evidence from the data that there is a distinct and important physiological value to a ration not measurable by present chemical methods or dependent upon mere supply of available energy. While the latter are important and give valuable data for a starting point, they are, nevertheless, inadequate as final criteria of the nutritive value of a feed.

Animals fed rations from different plant sources and comparably balanced in regard to the supply of digestible organic nutrients and production therms were not alike in respect to general vigor, size and strength of offspring and capacity for milk secretion.

Animals receiving their nutrients from the wheat plant were unable to perform normally and with vigor all the above physiological processes.

Those receiving their nutrients from the corn plant were strong and vigorous, in splendid condition all the time and reproduced young of large weight and vigor.

Animals receiving their nutrients from the oat plant were able to perform all the physiological processes of growth, reproduction

¹ Read by title

and milk secretion with a certain degree of vigor, but not in the same degree as manifested by the corn fed animals

Where a mixture of all the above plant materials was used the animals responded to the ration with less vigor than to the corn or oat rations alone, but with more vigor than to the wheat ration

These are records from the continued use of rations for three years. Monotony of diet was not a troublesome factor and is not of such importance in nutrition problems as usually supposed. The urines of the wheat-fed animals were acid to litmus. The urines from all the other lots were alkaline or neutral to the same indicator. Correction of this acid reaction by feeding alkaline carbonates did not restore the wheat fed group to full vigor and proper condition.

Allantoin was absent from the urines of the wheat group during their period of growth, but during gestation it was present. It was also present in the urines of the other lots.

The degree of oxidation of sulphur in the urines of the several groups was not greatly different.

At present we have no solution for the observations made. Differences of protein structure would appear to be excluded as a possible factor, because of the results secured with the mixed ration. Lack of adequate supply of bases in the wheat ration would also appear to be excluded as a factor, upon the basis of the records secured with the addition of inorganic ash mixtures for that ration. However, we reserve for the future all final conclusions as to the importance of these factors to our results.

SYNTHESIS OF LECITHINS IN THE HEN :

By E. V. McCOLLUM AND J. G. HALPIN

(From the Laboratory of Agricultural Chemistry, University of Wisconsin)

Young hens of about $2\frac{1}{2}$ pounds weight were fed a mixture of skim milk powder and rice meal, the latter twice extracted with a liberal amount of boiling alcohol. Ground limestone and sand were supplied. The ration was practically lecithin-free and nearly fat-free. During ninety days preceding the first laying period the hens increased their body weights from 33 to 36 per cent. An average egg production of 19 per hen was secured from birds continued on this ration.

The average weight of yolks was 15.5 grams, the total average yolk production per hen was 294.5 grams. By the method of Koch and Woods it was found that the average lecithin production was 8.83 grams, and of kephalins 27.68 grams per hen. It is fair to assume that the bodies of these hens contained more phosphatides at the end than at the beginning of the feeding period, and also that some phosphatides were present in the whites of the eggs. It is evident that the synthesis of phosphatides is readily accomplished in the body of the hen when fed rations free from these substances.

THE EFFECT OF HIGH MAGNESIUM INTAKE ON CALCIUM EXCRETION BY PIGS³

B. E. B. HART AND H. STEENBOCK

(From the Laboratory of Agricultural Chemistry, University of Wisconsin)

The theory that a high ratio of magnesium to calcium, which is normal to grains, is opposed to an optimum retention of calcium, has been studied. The work has been conducted with pigs and the data indicate that there is little validity for the theory. It has been shown by others that magnesium salts injected directly into the blood cause an increased excretion of calcium in the urine, our own work shows that magnesium salts excessively consumed with the food as chloride or sulphate, also occasion an increased excretion of calcium in the urine. When, however, these salts are accompanied by sufficient potassium phosphate to form the tertiary magnesium phosphate, the calcium excretion in the urine immediately decreases, further, when a high magnesium containing food, such as wheat bran or shorts, is ingested through the mouth, no increased calcium excretion in the urine takes place. This work indicates that faulty calcium retention for skeleton building, incident to feeding grains or grain by-products alone, does not lie in an improper ratio of these elements in the feed, but rather to a lack of supply of calcium. The fact that the relation of phosphorus to calcium and magnesium in our grains is high, with the probable formation in the tract of magnesium phosphate and its excretion by way of the intestine, would help explain this difference in the action of magnesium chloride or sulphate and the magnesium normal to grains.

³ Read by title

A COMPARISON OF THE NUTRITIVE VALUE OF THE NITROGEN OF THE OAT AND WHEAT GRAINS FOR THE GROWING PIG¹

By E. V. McCOLLUM

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

A young pig of 50 pounds weight was fed starch and salts alone for twelve days, then during sixty days a ration of rolled oats and starch which supplied 100 calories per kilo of body weight and fifty-five times the nitrogen daily eliminated by the pig as creatinine. This was followed by a ten-day starch period. This oat ration supplied about ten times the maintenance needs of the animal for nitrogen. Seven hundred and three grams of nitrogen were fed, of which 160.8 grams or 22.87 per cent was retained for growth.

In a like experiment with another pig the wheat grain and starch were given. The animal was fed per day fifty-five times the average nitrogen excreted daily as creatinine. Eight hundred and three grams of nitrogen was fed, of which 189 grams or 23.54 per cent was retained for growth. Both pigs were very vigorous specimens.

The data secured indicate that little if any difference exists in the utilization of the nitrogen of these grains by the pig for growth during a period of sixty days.

THE RELATION BETWEEN NITROGEN RETENTION AND RISE OF CREATININE EXCRETED DURING GROWTH IN THE PIG¹

By E. V. McCOLLUM

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

As previously reported (*Amer. Journ. of Physiol.*, **xxix**, p. 210) young pigs show a steady rise of creatinine output during growth which seemed to be proportional to the nitrogen retained. In two experiments a rise of 1 mgm. of creatinine nitrogen was found to accompany a retention of 2.39 and 2.46 grams of nitrogen. The pigs in these experiments were fed in one case casein, and in the other the proteins of skimmed milk.

In the experiments reported in the preceding abstract, the pig on a diet of rolled oats showed a rise of 0.0801 gram of creatinine

¹ Read by title.

nitrogen, during a period of sixty days in which 160.8 grams of nitrogen were retained. This corresponds to a rise of 1 mgm of creatinine nitrogen for each 2.01 grams of nitrogen retained.

In the wheat-fed pig a rise of 0.0740 gram of creatinine nitrogen was observed to accompany a retention of 189.0 grams of nitrogen. This corresponds to a rise of 1 mgm of creatinine per day for each 2.55 grams of nitrogen retained as growth. In experiments carried out with the great care exercised in the last two cases it was believed that closer agreement would be obtained in the results.

As a possible cause of the discrepancy in the figures obtained it may be suggested that certain protein mixtures may supply an abundance of complexes necessary to the formation of tissues concerned with creatinine formation, and a scarcity of complexes essential to the formation of tissues not so concerned. In other cases of restricted diet the reverse may be true. In other words, in the young animal, in which there is a great impetus to growth, if the proteins are derived from restricted sources there may occur an "unsymmetrical" growth. This question is being further investigated.

EXPERIMENTS IN FEEDING "DISSECTED" MILK⁵

B. E. V. McCOLLUM AND E. B. HART

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin)

A pig which weighed 17 pounds after a five-day fast, was fed 310.5 grams of nitrogen during fifty-five days. Seventy-five per cent was in the form of casein prepared by the method of Hammarsten, and 25 per cent in the form of boiled whey. Starch was given in amount sufficient to make the energy intake 75 calories per kilo per day. The pig excreted during this period 219.9 grams of nitrogen, leaving a positive balance of 90.6 grams. A significant rise in the creatinine output was observed. The body weight increased to 21.75 pounds. The experiment was discontinued because the pig became badly infested with worms.

A second pig weighing 22.5 pounds after a two-day fast, was fed 326.3 grams of nitrogen as casein during a fifty-five day period. The ration consisted of casein, starch, ash of whey, 2 grams of

⁵ Read by title.

agar-agar and water. The total nitrogen excreted during the period was 246.1 grams, leaving a balance of 80.2 grams retained. This pig was also found to be badly infested with worms and the experiment was discontinued. The weight at the end of the experiment was 23 pounds. Both of these pigs became very thin.

A third pig weighing 48 pounds was fed during sixty days a ration of skimmed milk and starch. The milk was treated each day with 86 per cent phosphoric acid to make 0.6 per cent acid, and boiled for fifteen minutes, cooled and treated with enough milk of lime to just neutralize it. The pig ate this mixture well. The animal retained 329 grams of nitrogen, and increased in weight 17 pounds. These experiments indicate that the pig can grow to a considerable extent on casein as the sole protein, and that milk treated so as to disturb any specific complexes between organic and inorganic radicals is still capable of maintaining a fairly vigorous growth.

THE URINE OF LATE PREGNANCY AND THE PUERPERIUM

By JOHN R. MURLIN AND H. C. BAILEY

(From the Departments of Physiology and Obstetrics, Cornell University Medical College, New York City)

From the maternity wards of the Bellevue Hospital and from the Emergency Hospital and School for Midwifery on 26th Street, were obtained continuous series of urines from the following cases:

1. Three normal pregnancies (ninth month) under control as to diet.
2. Three pre-eclamptic cases.
3. Two cases of eclampsia, one interpartum and one postpartum.
4. One case of pernicious vomiting with nephritis.

The normal cases on creatine-free diets containing less than 35 calories per kilogram show creatine in the urine. The percentage of ammonia nitrogen in the best-fed case ran as high as 12.2 per cent of the total. The amino-acid nitrogen by Henriques and Sørensen's method runs as high as 7.9 per cent.

The pre-eclamptic cases when placed on milk diet showed no high ammonia.

* Henriques and Sørensen *Zeitschrift f. physiol. Chem.*, lxxv, p. 120, 1910.

In the case of interpartum eclampsia, the ammonia was not above 6 per cent until after the convulsions. Afterwards it ran up to 30 per cent. The amino-acid N in this case just before labor was nearly 0.8 gram, being 6.6 per cent of the total. At the same time the undetermined nitrogen (possibly "peptid-bound" nitrogen) amounted to 3.7 per cent. In the other case of eclampsia the ammonia fraction was high in the first urine received by us, but fell rapidly as the patient's condition improved.

The single case of pernicious vomiting seems to bear out the views of Underhill and Rand⁷ as to the effects of starvation.

THE STORAGE OF FAT IN THE SALMON MUSCULAR TISSUE AND ITS RESORPTION DURING THE MIGRATION FAST ⁸

By CHAS. W. GREENE

(From the Department of Physiology and Pharmacology, Laboratory of Physiology, University of Missouri.)

The king salmon stores large quantities of fat in its tissues during its life in the ocean. When it enters the fresh waters of the rivers in the journey to the spawning ground it is now well known that it wholly ceases to take food and makes the journey while fasting. Food material is stored in the salmon tissues during the ocean feeding period and this food consists almost if not entirely of fat. In the Columbia River those salmon caught at the lowest point at the mouth of the river have the greatest amount of stored fat in their tissues.

The salmon fat is stored primarily in the muscles. These muscles are of three classes, namely, (1) The lateral dark muscle, (2) The great lateral pink muscle, (3) The small muscles of the fins and head. The fat is stored in each of these types of muscle in its own characteristic way.

1. The fat in the lateral dark muscle is in large drops chiefly within the fibers, but to some extent between the fibers. The fat drops between the fibers are relatively few in number and seldom exceed a diameter of 20 micra. The fat in the dark muscle within the fibers is in two characteristic regions, (a) between the sarco-

⁷ *Archives of Internal Medicine*, v, p. 61, 1910.

⁸ Abstract published by permission of the U. S. Commissioner of Fisheries.

lemma and the muscle plasma where the drops often amount to as much as 6 to 12 micra in diameter, and (b) within the sarcoplasm. This intramuscular fat is in unusually large droplets in size, often varying from 6 to 8 micra in diameter and located primarily in Cohnheim's areas. These larger droplets are associated with all sizes of fat droplets down to liposomes 2 micra in diameter.

The liposomes are in longitudinal chains located oftentimes between the individual fibrillae, the liposomes corresponding fairly closely in position and number with the striations of the muscle substance.

2 The fat in the pink muscle which represents the greatest muscular mass is wholly between the fibers, up to the time when the salmon stops feeding. The storage of fat in this region is enormous. The fat drops are of relatively large size, varying from the smaller ones only a few micra in diameter to drops as much as 100 micra in diameter.

3 In the smaller fin muscles which are in relatively constant activity, there is only a small amount of stored fat and that is chiefly intermuscular.

From the time the salmon stop feeding until their death after spawning the quantity of stored fat gradually diminishes. It is never wholly consumed even in fish taken after natural death. In the dark muscle the fat is gradually eliminated both from the inter and intrafibrous regions. It never wholly disappears from the substance of the muscle fiber but shows the extreme reduction at the time of dying. In the pink muscle the interfibrous fat is gradually removed during the migration period and has practically disappeared when the fish have reached the spawning stage and at the death which follows.

An observation of more than usual interest is found in the fact that a large quantity of extremely finely divided fat makes its appearance within the pink muscle fibers as soon as the fish stops feeding on entering the fresh water of the rivers. The fat is somewhat greater in amount and the droplets are slightly larger in the smallest fibers. This intrafibrous fat is present in all specimens at all stages of the migration journey. Its quantity is remarkably uniform. In fish from the spawning grounds which are approaching the spawning period this intramuscular fat begins to diminish in quantity. At the time of death, however, consider-

able quantities are still present in the smallest fibers though it has completely disappeared in the largest fibers

It seems evident that fat is thrown into the fibers of the great lateral muscle and kept there in strikingly uniform quantity and amount during the entire migration journey. It is suggested that this fat is utilized by the muscle as the source of the energy expended during the migration fast.

INTESTINAL ABSORPTION

By H C BRADLEY AND H S GASSER

(From the Laboratory of Physiological Chemistry, University of Wisconsin)

An emulsified mixture of olive oil and petroleum oil fed by sound to a dog leads to absorption of both fat and hydrocarbon. The thoracic lymph obtained by fistula contains both oils and in about the same relative proportion as in the emulsion fed. This suggests a mechanical absorption of droplets of fatty acid and hydrocarbon oil mixtures.

Isolated loops of the intestine of dogs, cats, and goats, were perfused with defibrinated blood from the same animal. The loops were either removed at the height of protein digestion or amino acid and peptone mixtures were introduced. Samples of the perfusing blood were taken at the beginning and at intervals during the experiment. Proteins from these samples were removed by mercuric nitrate, or phosphotungstic acid precipitation, or by coagulating in a boiling saturated solution of sodium or potassium sulphate. Tyrosine was not found in the concentrated filtrates from the protein precipitations although Millon's test is definite in dilutions of 1:100,000. No definite evidence of other amino acids could be found in the perfusate.

THE RELATIONSHIP OF THE SUPRARENAL GLANDS TO SUGAR PRODUCTION IN THE LIVER

By J J R MACLEOD AND R J PEARCE

(From the Laboratory of Physiology, Western Reserve Medical School, Cleveland)

That the failure of stimulation of the splanchnic nerve to produce evidence of hyperglycogenolysis, after removal of the corresponding adrenal gland, does not indicate that a hypersecre-

tion of adrenalin into the blood is the cause of the hyperglycogenolysis, which otherwise follows such stimulation, is shown by the fact that after complete section of the hepatic plexus splanchnic stimulation is usually without effect on the blood sugar

Further evidence of the direct nerve control of the process of hepatic glycogenolysis is that hyperglycaemia follows stimulation of the hepatic plexus

For this peripheral nerve control to be effective, however, there must be adrenalin in the blood, for, after double adrenalectomy, stimulation of the hepatic plexus is without effect on the blood sugar

METABOLISM IN AN EXPERIMENTAL FEVER WITH SPECIAL REFERENCE TO THE CREATININE ELIMINATION

By VICTOR C MYERS AND G O VOLOVIC

(From the Laboratory of Physiological Chemistry, Albany Medical College)

Fever was induced in rabbits (ten experiments) by inoculation with the bacillus of hog-cholera. Determinations of total nitrogen, urea, ammonia, creatinine, creatine, chlorides, potassium and phosphates, together with the morning and evening temperature observations, were made in the urine during the fever period and a previous control period of four or more days. The creatinine findings were of particular interest. The elimination of this substance during the fever was found to parallel very closely the body temperature, likewise the total nitrogen and urea, though the percentage of creatinine nitrogen in terms of total nitrogen dropped slightly at the height of the fever (3.8 to 3.3 per cent). The maximum temperature (about 42° C) was always found to be accompanied by the highest creatinine elimination, the percentage increase over the normal elimination averaging 36 per cent during this period. The elimination of creatine did not always accompany the fever, but when present was generally observed following the crisis of the disease. The view is expressed that the increased creatinine elimination still represents the normal endogenous protein metabolism which is proceeding at an abnormal intensity due to the increased temperature, while the presence of creatine suggests the exhaustion of the normal glycogen store of energy, and perhaps measures the amount of abnormal endogenous protein metabolism.

THE RÔLE OF PROTEINS IN GROWTH

BY THOMAS B OSBORNE AND LAFAYETTE B MENDEL

(From the Laboratories of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry of Yale University)

The proteins satisfy several functions in the growing organism. A certain minimum is necessary for the maintenance represented by Rubner's "Abnutzungsquote." With an additional adequate energy supply any excess of protein beyond this maintenance requirement may, in the adult, experience temporary storage or be devoted to dynamogenic uses, but in the organism capable of development it may contribute to growth.

The perfection of a product containing the non-protein constituents of milk (protein-free milk) in a form adapted to the specific needs of growing rats has made it possible to examine the efficiency of individual proteins in respect to maintenance and growth respectively. The investigations have indicated the inadequacy of all prolamines, viz., zein, gliadin, and hordein in contrast with efficient proteins such as casein, lactalbumin, ovalbumin, edestin, glycinn, and glutenin, in promoting growth. Gliadin and hordein satisfy the needs of maintenance in young animals, zein does not. It will be noted that all of the inadequate proteins are deficient in two or more familiar amino-acid complexes (Bausteine). Details of these experiments are presented in Publication 156, Part II, Carnegie Institution of Washington (1911).

THE RÔLE OF SURFACE TENSION IN THE DISTRIBUTION OF SALTS
IN LIVING MATTER

BY A. B. MACALLUM

(From the Laboratory of Biochemistry of the University of Toronto)

The author in previous communications to the Society had shown in a number of instances that the Gibbs-Thomson principle of surface concentration of solutes could reasonably account for the condensation of potassium salts found, by microchemical methods, to occur on certain surfaces and inclusions of living cells and structures, and he ventured to claim that this principle is the dominant force in determining the distribution of salts in living

matter In the present communication he brought evidence which definitely shows, in one group of instances, that this is the case In Marine Suctoria which, by alterations of surface tension of the superficial membrane or film at points on their surface, are able to protrude or retract tentacles formed of protoplasm, the distribution of potassium salts in the organisms is consequently affected When the tentacles are being protruded the potassium salts become localized in their films and very little may be found in the cytoplasm generally When the tentacles are being retracted the potassium salts begin to diffuse throughout the cytoplasm, where it remains until it is excreted, or until the tentacles are again being protruded, when it is once more condensed in the excessively thin surface films of the tentacles

A COMPARISON OF THE EFFECTS OF SUBCUTANEOUS AND INTRAMUSCULAR INJECTIONS OF ADRENALIN UPON THE PRODUCTION OF GLYCOSURIA

By I S KLEINER AND S J MELTZER

(From the Rockefeller Institute for Medical Research)

The experiments were made on rabbits, all of which received no food for twenty-four hours previous to the injection but received 100 cc of water by stomach tube shortly before the injection of adrenalin The urine was collected for twenty-four hours after the injection The intramuscular injections were made into the lumbar muscles, subcutaneous, in the lower part of the abdomen The doses of adrenalin ranged from 0.3 to 1.0 cc of 0.1 per cent solution In forty-nine rabbits the average amount of sugar eliminated following intramuscular injection was 0.73 gram in forty-nine animals receiving like doses by subcutaneous injection, the average sugar excretion was 1.20 gram A difference in favor of the subcutaneous injection was noted for every dose tested, though the difference grew less as the dose diminished The greatest differences were observed with 0.7 or 0.8 cc of adrenalin solution Of the forty-nine intramuscular injections, thirteen were not followed by glycosuria of the forty-nine subcutaneous injections, only four failed to cause glycosuria

In eight experiments, a dose of 0.75 cc of adrenalin solution was injected subcutaneously distributed over four different places

Four of the animals excreted no sugar the average sugar excretion of the other four was only 0.56 gram The average excretion of sugar following the subcutaneous injection of this dose at a single point was 1.52 grams

The experiments show that methods which favor the absorption of other substances are less favorable for the production of glycosuria by adrenalin

THE HOURLY CHEMICAL AND ENERGY TRANSFORMATIONS IN THE DOG, AFTER GIVING A LARGE QUANTITY OF MEAT

By H. B. WILLIAMS, J. A. RICHE AND GRAHAM LUSK

(From the Physiological Laboratory, Cornell Medical College)

A calorimeter of the Atwater-Rosa type was constructed by Dr Williams, which is capable of measuring with great accuracy the heat of combustion of alcohol and the oxygen absorbed and the carbonic acid produced during its combustion, during periods of one hour each

A dog which had been fed 700 grams of meat at noon of the previous day, was placed in the calorimeter between 10 and 11 o'clock in the morning, and his metabolism measured The animal was given 1200 grams of meat at noon and placed in the apparatus again The heat production and other factors of metabolism were determined during hourly periods for twenty hours

1 It was found that the direct and the indirect calorimetry agreed perfectly

2 It was found that the heat production rose largely, and that this increase in heat production was proportional to the nitrogen eliminated in the urine and was in no way proportional to the quantity of material present in the intestine

3 It was found that the carbon which was retained from the protein ingested, must have been retained in the form of glycogen, since the absorption of oxygen during the different periods corresponded exactly with this assumption, whereas, if the carbon had been retained in the form of fat, the oxygen absorption would have been 10 per cent less than that found

Further experiments have shown that glutamic acid added to a standard diet does not increase the heat production in any way

CHEMICAL ANALYSES OF THE ASH OF SMOOTH MUSCLE

BY L. A. RYAN AND EDWARD B. MEIGS

(From the Wistar Institute of Anatomy and the Hare Chemical Laboratory of the University of Pennsylvania)

The ash of the smooth muscle of the bull-frog's stomach has been analyzed for potassium, sodium, phosphorus, and chlorine. The methods of analysis have been in general those described by Katz in the *Archiv für die gesammte Physiologie*, 1896, lxxii, p. 1, and the striated muscle of the same frogs was analyzed by the same methods for the same elements. The quantities of the elements found in the striated muscle were about the same as those reported by Katz in the article mentioned above. In the smooth muscle three analyses were made for potassium, sodium, and chlorine, and four for phosphorus. The following quantities, given as percentages of the weight of the fresh muscle, were obtained

	I	II	III	IV	AVERAGE
	per cent	per cent	per cent	per cent	per cent
Potassium	0.306	0.343	0.346		0.332
Sodium	0.051	0.065	0.050		0.055
Phosphorus	0.128	0.133	0.146	0.149	0.139
Chlorine	0.099	0.120	0.121		0.117

Samples of the tissue analyzed as "smooth muscle" were examined microscopically, both in the fresh state and after fixation by various histological methods, and it was determined that from 70 per cent to 85 per cent of the volume of the tissue was made up of smooth muscle fibers, about 5 per cent was extraneous connective tissue, and the remainder, interstitial spaces between the muscle fibers.

The results of our investigation indicate that smooth muscle contains somewhat less potassium and phosphorus and somewhat more sodium and chlorine than the striated muscle of the same animal, but that the differences in these respects between the two tissues are not by any means so marked as has sometimes been supposed.

QUANTITATIVE MEASUREMENT OF OXIDASES

By H H BUNZEL

(From the Bureau of Plant Industry, U S Department of Agriculture)

Accurate measurements were made of the oxidizing power of potato juice towards a series of aromatic substances, such as tyrosine, benzdin, hydrochinone, α -naphthol, guaiacol, and others, and a comparative study of the behavior towards these substances was made. Experiments are described on the susceptibility of the oxidases toward poisons and heat. The fact that the oxidizing power of the juice is limited, and the reaction comes to completion after several hours, as found in the case of pyrogallol, has been confirmed for about half a dozen other substances, as has also the fact that the extent of the oxidation is directly proportional to the quantity of enzyme used. An entirely new fact has been brought out. The oxidizing power of the juice towards different substances to be oxidized is not additive. If one uses two or three oxidizable materials in the same experiment, the result is not a summation of the individual oxidations where the oxidation by the same juice is measured separately, but corresponds roughly to the result obtained in the case of the most rapidly oxidized substance.

THE ESTIMATION OF DEXTROSE IN BLOOD AND URINE BY THE DIFFERENCE IN REDUCING POWER BEFORE AND AFTER YEAST FERMENTATION

By J J R MACLEOD, C D CHRISTIE AND J D DONALDSON

(From the Physiological Laboratory, Western Reserve University)

After treatment of urine with 10 per cent blood charcoal (Merck) in the presence of 15 per cent acetone or 25 per cent glacial acetic acid, we have not found any adsorption of added dextrose to occur.⁹ When the reducing power of such clarified urine is estimated by Bang's method before and after twenty-four hours fermentation with fresh brewers' yeast there is not infrequently more reduction after fermentation than before.

⁹ Cf Woodyat and Helmholtz *Journ of Exp Med*, vii, p 598, 1910, Andersen *Biochem Zeitschr*, \\\vii, p 262, 1911

Before	After
0 083	0 072
0 039	0 042
0 087	0 062

It is therefore impossible to estimate the amount of dextrose in urine by the difference in reducing power, as estimated by Bang's method, before and after fermentation

These results raised the suspicion that yeast might produce some substance which, although not sugar, caused reduction with Bang's solution

We have therefore compared the reducing power as estimated by Bang's, Allihn's and Bertrand's methods of (1) a 10 per cent suspension of yeast in water, (2) a 10 per cent suspension of yeast in dextrose solution and have found that there is always some reduction by Bang's method but none, or the merest trace by the other methods For example, one per cent dextrose solution after fermentation for twenty-four hours gave 0 07 per cent dextrose (?) (Bang) and 0 002 (Allihn)

These results probably explain the high residual reduction found by Lyttkens, *et al*, in blood after yeast fermentation, and from which they conclude that a considerable proportion of the reducing substance in blood is other than dextrose Thus in blood plasma (dog) we have found

Percent dextrose before fermentation	{ Bang	0 166
	{ Bertrand.	0 165
Percent dextrose after twenty-four hours fermentation	{ Bang	0 060
	{ Bertrand	trace

A NEW METHOD FOR THE DETERMINATION OF HIPPURIC ACID IN URINE

By OTTO FOLIN AND FRED F FLANDERS

(From the Laboratory of Biological Chemistry, Harvard Medical School)

The method is based on the hydrolysis of hippuric acid, the extraction of the benzoic acid with chloroform and titration with sodium ethylate ¹⁰

To 100 cc urine add 10 cc normal NaOH Evaporate to dryness on steam bath Boil the residue 4½ hours in a 500 cc Kjeldahl

¹⁰ Journ of the Amer Chem Soc, xxviii, p 161, 1911

flask, fitted with Hopkins condenser, with 25 cc of water and 25 cc of concentrated HNO_3 . Dilute to 100 cc, saturate with ammonium sulphate and extract with 50, 35, 25 and 25 cc portions of chloroform. Shake the extract with 100 cc of saturated NaCl containing 0.5 cc of concentrated HCl per liter. Separate and titrate with $\frac{N}{10}$ sodium ethylate. The method gives theoretical results on pure hippuric acid and excellent duplicates on urine.

SYNTHETIC ACTION OF ENZYMES

By H. C. BRADLEY

(From the Laboratory of Physiological Chemistry, University of Wisconsin)

A comparison of the fat content and lipase activity of various vertebrate livers showed no apparent relation between the two. Fish livers which are evidently active in the storage and utilization of fat, may contain ten times as much fat as mammalian liver, on the other hand the latter may be nearly ten times as active lipolytically as the former. A quantitative comparison of lipolytic activity appears of doubtful value in supporting the theory of the synthetic function of enzymes in tissues.

Mammary glands from goats and other animals taken at the height of lactation have thus far failed to show the presence of lactase, the enzyme which should synthesize as well as hydrolyze lactose. Lipase of the active gland is much less abundant than in the liver of the same animal. The autolytic ferments of mammary and liver tissues are about equal as measured by the rate of digestion of a sodium caseinate solution. It has thus far been impossible to secure definite evidence of the synthetic function of enzymes in living tissues.

NOTE ON THE INORGANIC CONSTITUENTS OF HUMAN BLOOD¹¹

By C. C. BENSON

(From the Laboratory of Biochemistry, University of Toronto)

Whole blood and serum were analyzed for sodium, potassium, magnesium, calcium and chlorine, using modern methods. The results of analyses show slight variations from earlier analyses.

¹¹ Read by title.

ON SPHINGOSIN ¹.

BY P A LEVENE AND W A JACOBS

(From the Rockefeller Institute for Medical Research)

Sphingosin was discovered first by Tudichum on hydrolytic cleavage of phrenosin. It was rediscovered later by Thierfelder on decomposition of cerebrin. Regarding the chemical structure of the base there existed very little information beyond the knowledge of its empirical formula. This was presented as $C_{17}H_{35}N_2O_2$. The present work was undertaken with a view of elucidating the structure of the base. The work is still in progress, but the data already obtained lead to the conclusion that sphingosin is an unsaturated amino-alcohol of the olefine series.

The data on which the conclusion is based are the following. The substance contains all its nitrogen in form of primary amino-nitrogen, it forms a triacetyl derivative, which no longer contains the original primary amino group. It absorbs hydrogen in a proportion equivalent to one unsaturated bond. The substance obtained by the last process has the composition of dihydrosphingosin, $C_{11}H_{37}NO_2$. It was analyzed as its triacetyl derivative.

In connection with this mention must be made of the fact that the substance obtained by Thierfelder in the filtrate from sphingosin sulphate and which is described as a nameless base, is in fact dimethylsphingosin, which is formed in the process of preparing sphingosin. This view is based on the results of the determination of the methyl groups present in the molecule of the nameless base.

ON GLYCOLYSIS ¹

BY P A LEVENE AND G M MEYER

(From the Rockefeller Institute for Medical Research)

The work of the previous year brought to light the facts that the action of tissue extracts on glucose was either altogether negative, or consisted in a condensation of the monosaccharide into a more complex form. Thus, the problem of the study of glycolysis had not been furthered by those experiments, and it remained

¹Read by title

necessary to discover a method by which could be studied the chemical process of sugar degradation within the living tissues

It was concluded to employ for these experiments leucocytes obtained under aseptic precaution and to perform the experiments under perfectly aseptic conditions. There was noted a marked fall of the reducing power of a sugar solution kept in contact with the leucocytes. After the action of the leucocytes the sugar solution could not be brought to its original reducing power by boiling with mineral acids.

The analysis of the products of glycolysis under such conditions revealed the absence of carbonic acid, or of any volatile acids. The only substance obtained from the reaction mixture was lactic acid. This was identified as its zinc salt. The value of the lactic acid obtained in the experiments was lower than that of the sugar decomposed by the leucocytes. Control experiments with leucocytes failed to discover lactic acid. Thus, it is for the first time definitely established that lactic acid is an intermediate product of glycolysis.

ON THE PICRATE OF GLYCOCOLL ¹³

B₁ P. A. LEVENE AND D. D. VAN SLYKE

(From the Rockefeller Institute for Medical Research)

In a previous publication by one of us (L.) the picrate of glycoll was described having the composition of $C_2H_5NO_2 \cdot C_6H_3(NO_2)_3$, and the $M.P. = 190^\circ C$. The present investigation brought to light the fact that this substance was a mixture of glycoll picrate of the composition $(C_2H_5NO_2)_2 \cdot C_6H_3(NO_2)_3$, and of picric acid. Diglycoll picrate is a very stable salt, which can be recrystallized without altering its composition and its melting point. The melting point of diglycoll picrate = $201^\circ C$ (corrected), on the other hand it is possible to prepare artificial mixtures of the picrate and of picric acid which melt as $191^\circ C$.

¹³ Read by title

A METABOLISM STUDY ON A FASTING MAN

BY PAUL E. HOWE AND P. B. HAWK

(From the Laboratory of Physiological Chemistry, University of Illinois)

A second seven-day fast on "Subject E" was reported. The conditions of experimentation were as follows, a preliminary period, of high protein ingestion, 21.86 grams of nitrogen per day, a fasting period with constant water ingestion, a low protein feeding period, 5.23 grams of nitrogen per day for four days, and a final high protein feeding period, 21.86 grams of nitrogen per day for five days. Observations were made on the changes in body weight and the total nitrogen, urea, ammonia, creatinine and creatine excretions. The phosphates, chlorides, acidity and hydrogen ion concentration of the urine were also determined but not reported. Data on indican, ethereal sulphates and fecal bacteria were reported in another connection.

The effects produced by the ingestion of a high protein diet previous to the fast as compared with those obtained in the previous fast which was preceded by a low protein period, were, a high rate of protein decomposition during the fast with an unusually high nitrogen excretion on the third day, a higher creatinine nitrogen excretion for the first three days of the fast which approximated the creatinine excretion of the first fast for the last four days, the same percentage relations between the total nitrogen and urea nitrogen excretions, a smaller loss of body weight.

During the feeding period there were negative nitrogen balances with slight gains in weight during the low-protein feeding period and positive nitrogen balances and marked gains in weight for the high protein feeding period. There was a gain in weight of 4.11 kg. in eight days with a nitrogen retention of but 4.52 grams on an intake of 108.2 grams of nitrogen as contrasted with a gain of but 3.08 kg. in weight and a retention of 25.20 grams of nitrogen on an intake of 139.3 grams of nitrogen for the first fast. The data indicate that this gain was largely of non-nitrogenous substances other than water. If we accept the percentage distribution of nitrogen as a criterion of normal metabolism there was a return to such a condition on the fourth day of feeding.

HYDROGEN ION CONCENTRATION OF FECAL EXTRACTS

B₁ PAUL E. HOWE AND P. B. HAWK*(From the Laboratory of Physiological Chemistry, University of Illinois)*

The acidity of fecal extracts was determined during a series of experiments upon the effect of water-drinking with meals and during a seven-day fasting period and the subsequent feeding periods which consisted of a low- and a high-protein period. The general type of the diet was the same in all cases. The Salm type of hydrogen electrode cell was used. The feces were extracted, by means of centrifugation, with 0.5 normal solution of Na_2SO_4 . A mixture of 0.2 mole of Na_2HPO_4 and 0.1 mole of NaH_2PO_4 was used as the standard of comparison. A two gram sample of moist feces was used in each instance.

The hydrogen ion concentration of the fecal extracts did not show any pronounced changes as the result of the ingestion of increased amounts of water, with meals—the results varying between 1×10^{-8} and 0.1×10^{-8} mole of hydrogen ion per liter. As the result of fasting there was in general a decrease in the hydrogen ion concentration, from an average of 5.3×10^{-8} for the normal fasting period to 1.2×10^{-8} for the two fasting stools. The hydrogen ion concentration is different for different individuals on the same diet but in general rather uniform for each individual.

CONNECTIVE TISSUES OF LIMULUS¹⁴B₁ H. C. BRADLEY*(From the Laboratory of Physiological Chemistry, University of Wisconsin)*

A chemical study of the cartilage-like connecting tissues of the gill plates of *limulus*, and the fibrous and tendon-like tissues to which the pedal muscles are attached.

THE RESPIRATION CALORIMETER AND ITS USES FOR THE STUDY OF PROBLEMS OF VEGETABLE PHYSIOLOGY ¹⁵

BY C F LANGWORTHY AND R D MILNER

(From the Office of Experiments Stations, U S Department of Agriculture)

In reconstructing the Atwater respiration calorimeter which was transferred at the death of Professor Atwater from Middletown to the laboratory of the Department of Agriculture, improvements were introduced in the devices for controlling the temperature of the water flowing through the calorimeter, which carries out the heat generated in the chamber, and for automatically recording the difference in temperature of the water as it enters and as it leaves the chamber

This calorimeter has recently been applied to a new line of investigations concerned with the ripening of fruit Several bunches of bananas were placed in the chamber and kept under observation during ripening, the oxygen consumption, carbon dioxide excretion and heat elimination being determined The data obtained indicate that physical and chemical factors of both theoretical and practical value may be measured with the respiration calorimeter, and afford evidences of the adaptability of this instrument to the study of fundamental problems of plant life

A new respiration calorimeter especially constructed for the study of the problems here alluded to is nearly completed in which the size of the chamber is reduced and in which such recording and controlling devices have been introduced as to make the apparatus nearly automatic in its operation

ON THE EXCRETION OF FORMALDEHYDE, AMMONIA AND HEXAMETHYLENAMINE ¹⁵

BY HUGH McGUIGAN

(From the Laboratory of Pharmacology, Northwestern University Medical School)

When formaldehyde is injected intravenously it is oxidized with surprising rapidity One hundred cubic centimeters of 1 per cent formaldehyde, injected into a 10 pound dog in the course of one

¹⁵ Read by title

and one half hours, completely disappeared from the blood within thirty minutes after the injection. Only formic acid was present in the urine. Formaldehyde is also excreted into, but less rapidly oxidized in, the intestine. In other instances free formaldehyde was found in the urine, only when large doses were given.

A like amount of hexamethylamine, injected in the same manner, could be found in the blood several hours after the injection. It was also found (formaldehyde test) in the urine, bile, intestines, eye, saliva, bronchial secretions, amniotic fluid, eggs (hen) and sweat (human).

Free formaldehyde is at least much harder to detect in these fluids. The tests in most cases were negative.

Ammonia is not excreted by the lungs. Combined with formaldehyde, however, it is found in the bronchial secretions. From the similarity of the alkaloids to ammonia it was thought that, perhaps morphine, which is not excreted normally by the kidneys, might pass through if administered with formaldehyde. No positive result on this point has been obtained.

GLYCOLYSIS, AS MODIFIED BY REMOVAL OF THE PANCREAS AND BY THE ADDITION OF ANTISEPTICS ¹⁶

By H. McGUIGAN AND C. L. VON HESS

(From the Laboratory of Pharmacology, Northwestern University Medical School)

Repetition of previous work showed that mixtures of extracts of normal muscle and of pancreas, with toluol or chloroform added as an antiseptic, caused no or only slight glycolysis, but not more than normal muscle extract alone.

The above suggestions are open to two criticisms.

1 (Suggested by Woodyatt, also by Oppenheimer) if, by Cohnheim's theory, there be a pancreatic internal secretion, the normal muscle would probably contain enough of it to exert maximal glycolysis, hence no change will be obtained by the addition of pancreatic extract. However, muscle of pancreatectomized dogs, similarly tested, gave no action on glucose either with or without the addition of pancreatic extract.

¹⁶Read by title

2 Aseptic glycolysis of yeast or of blood is greatly inhibited by antiseptics in the concentrations used in the muscle experiments. The foregoing method, which involves the use of antiseptics, destroys normal glycolysis to such a degree that the results obtained by it can prove neither the presence nor the absence of an internal secretion of the pancreas. It further indicates that normal glycolysis is due more to cellular than to enzyme activity.

EFFECT OF THE QUANTITY OF PROTEIN INGESTED ON THE NUTRITION OF ANIMALS VI ON THE CHEMICAL COMPOSITION OF THE ENTIRE BODY OF SWINE ¹⁷

By A. D. EMMETT, W. E. JOSEPH AND R. H. WILLIAMS

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois)

Three lots of young pigs, four in a lot, were fed on low, medium, and high protein planes. One pig of the low protein lot and two from each of the medium and high protein lots were subjected to detailed slaughter tests as soon as the medium or standard fed pigs reached marketable weight and condition. The various parts of the animals were analyzed.

It was found (I) That, out of the four pigs kept on the low protein plane, three died before the close of the experiment. These pigs grew slowly, were drowsy, lacked vigor and became stiff in their joints. The pigs of the other two lots were thrifty, in good condition and grew normally. Blood counts showed no definite differences. (II) That the average daily gains for the 174 days of individual feeding were 0.33, 0.85, 0.90 pounds respectively for the low, medium, and high fed lots. From the standpoint of economy of gains, the medium protein-fed lot made the best showing. (III) That the chemical data for the entire bodies of the five slaughtered pigs showed the medium and high protein-fed lots to be remarkably similar in their percentages of water, fat, protein, ash, and phosphorus. The pig of the low protein lot had a low percentage of fat and a high one of ash and phosphorus. Comparing the data from the five animals with the average of two representative pigs, slaughtered at the beginning of the experiment,

¹⁷ Read by title.

the percentage increase of dry substance, protein, fat, ash, and phosphorus was lowest in the low protein-fed pig. In case of the medium and high protein-fed pigs the percentage of increase of the nutrients was practically the same. In the majority of instances the differences between the averages within the lots were greater than those between the averages of the lots. (IV) That the average chemical composition of the bodies of the five pigs, at about 200 pounds live weight and in good marketable condition, was on the fresh basis: water, 45.74, protein, 14.38, fat, 37.20, ash, 3.84, and phosphorus, 0.673 per cent.

THE EFFECT OF QUININE ON CULTURES OF PNEUMOCOCCI¹⁸

A PRELIMINARY REPORT

By O. H. BROWN

(From the St. Louis University School of Medicine)

Numerous reports of apparent specific curative effects of large doses of quinine in pneumonia led me to use it in a small number of cases of this disease. While the results were highly satisfactory, I afterward concluded that they were probably accidental because of the apparently trustworthy claims made by other clinicians that quinine failed to show any beneficial result in their cases.

I have however carried out tests upon the antiseptic power of quinine and its salts of citric, sulphuric and salicylic acids upon pneumococci *in vitro*. Sterile tubes of human blood bouillon were prepared, half of them containing quinine in the form and percentage (0.05-0.1 per cent) which I desired to test. Inoculations into plain bouillon (control) and into quinine bouillon were made. Plate cultures on blood agar made immediately and afterward at varying intervals showed the growth or destruction of pneumococci in such tube. Thirty strains of pneumococci from various sources have been tested. The results showed (1) Pure quinine is more destructive to pneumococci than are its salts. (2) The time required for 0.1 per cent quinine to kill pneumococci varies from twenty minutes to four or five hours. (3) Other organisms, such as streptococci and staphylococci, are destroyed only by much longer exposure to quinine.

¹⁸ Read by title

MAINTENANCE AND GROWTH

BY THOMAS B OSBORNE AND LAFAYETTE B MENDEL

(From the Laboratories of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry of Yale University)

In connection with the authors' feeding experiments with isolated food-substances it has been found that diets which are satisfactory for the maintenance of full-grown animals are entirely inadequate to induce growth in ungrown individuals. The suspension of growth on a maintenance diet here referred to is not that caused by an insufficient supply of energy, but is a retarded development associated with the chemical make-up of the diet. These chemical features of the diet essential for proper growth involve not only the type of protein, but likewise certain non-protein components (presumably the inorganic ingredients). Dwarfing, in the sense of maintenance of *both weight and size*, can readily be brought about in young animals, and the capacity to grow can be maintained unimpaired by such stunted individual for many months. The non-protein constituents of the diet can be prepared from the protein-free portions of cow's milk (protein-free milk) in a form suitable to permit proper growth. The experimental records of rats, selected as the animals for study because they manifest the utilization of a suitable diet speedily by measurable changes in size, are presented in Publication 156, Part II, Carnegie Institution of Washington (1911).

THE STUDY OF ENVIRONMENT

BY WILDER D BANCROFT

(From the Department of Physical Chemistry, Cornell University)

When studying the effect of environment on an organism, we must distinguish three distinct things: the direct effect of new external conditions involving no adaptation, the adaptation of the organism to the new conditions, and the possible inheritance of the adaptations. The botanists have not made these distinctions. They consider the change of curvature of tendrils with change of temperature as a case of non-adaptive response, whereas it has no more to do with adaptation than the shortening of a fishing-line when it is wetted.

The problem of the inheritance of acquired characters has been complicated unnecessarily by the arbitrary limitation that the character must be inherited for four or five generations after the organism has been brought back to the original surroundings. Since an organism which responds readily to a new environment will also revert readily when brought back, this definition has probably excluded most of the cases in which the inheritance of acquired characters could be shown. The biologists seem never to have realized that inheritance is primarily a hysteresis phenomenon and should be studied as such.

THE SYNTHESIS OF THIOTYROSINE

By TREAT B. JOHNSON

(From the Sheffield Laboratory of Yale University)

A knowledge of this new amino-acid was especially desirable, in order to acquire a more definite conception of the true nature of sulphur combinations in proteins. The acid has been prepared by the application of a new, general method for the synthesis of α -amino acids and its chemical properties are now being studied.

The most important characteristic of the acid, so far observed, is the fact that it does not give Millon's test. On the other hand, it gives, on warming the concentrated sulphuric acid, as characteristic color reaction as the Millon's test is characteristic for tyrosine. This study is one of a projected series on new sulphur combinations which has been planned for the Sheffield Laboratory.

THE RELATION OF OHIO BOG VEGETATION TO THE CHEMICAL NATURE OF PEAT SOILS

By ALFRED DACHNOWSKI

(From the Department of Botany, Ohio State University)

Analyses are submitted showing that several types of vegetation of varied growth-form occur upon a habitat essentially similar in range of chemical composition. The prime conditions determining distributional relationships and succession are not the mineral salts in the soil but biochemical processes. The variable composition of peat renders it necessary to determine experimentally what organic substances are absorbed and of value or injurious in nutritive metabolism.

PHYTOCHEMICAL STUDIES IN CYANOGENESIS

B. C. L. ALSBERG AND O. F. BLACK

(From the Bureau of Plant Industry, U. S. Department of Agriculture)

The relation between the nitrates in the soil, nitrification during drought, and cyanogenesis in sorghum, based on experiments done at the Arlington Farm in the course of the past summer, is discussed, and an incidental error in the common method of determining hydrocyanic acid in plants is pointed out.

THE NITROGEN EXCRETION OF THE MONKEY, WITH SPECIAL REFERENCE TO THE METABOLISM OF PURINES

B. ANDREW HUNTER AND MAURICE H. GIVENS

(From the Department of Physiology and Biochemistry, Cornell University)

A female monkey (*Cercopithecus callitrichus*), weighing 4.7 kilograms, was maintained for forty days on a daily ration of 200 cc whole milk, 200 grams bananas, and 20 grams peanuts. The urine was collected every forty-eight hours. For the first sixteen days the average daily excretion of N was 1.83 grams, distributed as follows: urea, 1.59, NH_3 , 0.028, creatinine, 0.065, allantoin, 0.015, purines, 0.0027, undetermined, 0.13 grams N, or, urea 86.9, NH_3 , 1.5, creatinine, 3.5, allantoin, 0.82, purines, 0.15, undetermined, 7.1 per cent of total N. Uric acid could not be detected.

During the remainder of the experiment attention was devoted particularly to the metabolism of endogenous and exogenous purines. On seven normal two-day periods the excretion of allantoin N ranged from 27.0 to 31.8, that of purine N from 4.7 to 10.3 mgs. On five periods, each interpolated between two normal ones, doses of 0.5, 0.5, 1.0, 1.0, and 2.0 grams sodium nucleate were administered. Of the purine N thus fed 90, 56, 41, 24, and 29 per cent respectively of the theoretically possible was recovered in the form of allantoin and urinary purines. Of the amount so recovered 79 to 98 per cent took the form of allantoin, after the second dose of 0.5 grams 2 per cent, and after 2.0 grams 9 per cent appeared as uric acid. In normal periods allantoin accounted for 71–87, in nucleate periods 77–86, per cent of the total purine-allantoin N. In respect of the ratio between allantoin and purine excretion the

species examined resembles the lower mammals rather than man. On the other hand we did not meet with the almost quantitative conversion of exogenous purines into allantoin, which has been reported for the dog.

THE DEFINITION OF NORMAL URINE ¹⁹

By JOHN H. LONG

(From the Laboratory of Physiological Chemistry, Northwestern University Medical School)

Our notions as to what is a normal urine have undergone many changes in the years which have elapsed since the first attempts were made to establish standards. The same individual, at one time on a high protein diet and again on a low protein diet, will excrete urine which may be markedly different in many ways, and yet both be normal.

Improved methods of examination have shown that hyaline casts are much more frequently present in the urine of healthy men than was suspected a few years ago, and it must be admitted that traces of albumin occur in the urines of men, who, from all ordinary points of view are perfectly well.

The statement as to what constitutes normal urine must take cognizance of these facts and of the further fact that for each individual there seem to be agencies at work which modify the nitrogen distribution, the acidity and the neutral sulphur in ways which we cannot account for. In a certain sense each individual has his own standard of normality.

SHOULD THE TERM PROTAGON BE RETAINED

By WALDEMAR KOCH

(From the Laboratory of Physiological Chemistry and Pharmacology, University of Chicago)

Data were presented which indicated that the preparations referred to as protagon contain at least three substances: a phosphatid containing cholin, a cerebrosid-containing sugar, a complex combination of a cholin-free phosphatid with a cerebrosid to which an ethereal sulphuric acid group is attached. The term protagon cannot therefore be said to have any chemical significance. The details will be presented in a more extended publication.

¹⁹ Read by title.

OXIDIZING ENZYMES IN CERTAIN FUNGI PATHOGENIC FOR PLANTS

BY H S REED AND H S STAHL

(From the Laboratory of Plant Pathology, Virginia Agriculture Experiment Station, Blacksburg, Virginia)

The oxidizing ability of the plant extract is often altered as a result of the invasion of parasitic fungi. The extracts of apples invaded by *Sphaeropsis malorum* show no oxidizing powers whatever. Apples attacked by *Glomerella ruformaculans* show on the contrary a somewhat increased oxidizing ability. When grown in pure culture on synthetic media *Glomerella* develops oxidizing enzymes in certain media but not in others.

MODIFIED COLLODION MEMBRANES FOR STUDIES OF DIFFUSION *

BY WILLIAM J GIES

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York)

Lipins and many substances which dissolve in ether, alcohol and similar solvents can be dissolved, in large proportions, in U S P collodion solution without inducing precipitation of the collodion. Such mixed solutions, when treated in any of the usual ways for the production of collodion membranes, yield composite homogeneous products. If the proportion of added substance is not too large it is wholly incorporated uniformly in the resultant composite membrane. Lecithin, cholesterol, lard, olive oil, rubber, alcohol-ether soluble protein, organic pigments, ferric sulfocyanate and many other substances have been incorporated homogeneously in such modified collodion membranes. Membranes prepared in this way show interesting differences in permeability in diffusion experiments, according to the general nature of the incorporated materials. Such membranes promise to afford valuable means of studying cell permeability and osmosis in general under biological conditions. I am proceeding with various types of experiments with such modified collodion membranes in the hope of extending our knowledge in these particular directions.

* Read by title

A METHOD FOR DIFFERENTIATING BETWEEN "METABOLIC" AND RESIDUAL FOOD NITROGEN OF THE FECES ²¹

By MORRIS S FINE

(From the Sheffield Laboratory of Physiological Chemistry, Yale University)

It is hardly necessary to point out that the nitrogen of the feces is in great part composed of bacteria, unabsorbed intestinal secretions, etc. If the quantity of this "metabolic" nitrogen were known, the nitrogen of the food actually escaping absorption could readily be estimated. Investigators have sought a measure of the "metabolic" nitrogen in the feces obtained during starvation or from a digestible non-nitrogenous diet, or the attempt has been made to differentiate by chemical means. As a rule, such methods do not take into account the fact that the indigestible materials, e g, cellulose and hemicellulose such as are present in cereals, legumes, etc, show a marked tendency to increase the elimination of fecal material. That this is a consideration of some importance is shown in a paper from this laboratory, now in press. The following procedure is believed to offer certain advantages over those hitherto proposed. From the fecal nitrogen accruing from a given diet is subtracted the corresponding value resulting from a *non-nitrogenous diet, yielding practically the same amount of feces*. Such a non-nitrogenous diet may be conveniently obtained by adding agar-agar to non-nitrogenous food whose calorific equivalent does not differ materially from that of the diet under investigation. The result thus obtained represents the amount of nitrogen of the latter diet which has escaped utilization.

BIOCHEMICAL AND BACTERIOLOGICAL STUDIES OF THE BANANA ²¹

By E MONROE BAILEY

(From the Connecticut Agricultural Experiment Station)

An earlier study²² has been extended. Enzymes concerned in ripening processes have been investigated, and in addition, bacteriological and chemical examinations of the fruit in various stages of maturation have been made. Amylase, sucrase, raffinase, pro-

²¹ Read by title

²² *Journal of Biological Chemistry*, 1, p 355, 1906

tease, lipase, and peroxidase were detected Tests for maltase, dextrinase and lactase were doubtful or negative The inner portion of the pulp of sound fruits appears to be sterile, but the regions of the inner coats of the peel may be sparsely inhabited by bacteria As ripening progresses, starch disappears and the content of alcohol-soluble sugars and dextrine increases Maltose could not be detected

PREPARATION OF CREATINE AND CREATININE FROM URINE

By STANLEY R. BENEDICT

ESTIMATION OF CREATININE

By STANLEY R. BENEDICT

CREATINE ELIMINATION IN THE PREGNANT DOG

By J. R. MURLIN AND H. I. MULLER

THE IODINE CONTENT OF THYROID GLANDS OF SHEEP FED MAINLY UPON MARINE ALGAE

By ANDREW HUNTER AND SUTHERLAND SIMPSON

RECOVERY OF ALCOHOL FROM ANIMAL TISSUES

By P. J. HANZLIK²

CHANGES IN THE COMPOSITION OF BLOOD AND MUSCLE FOLLOWING DOUBLE NEPHRECTOMY AND BILATERAL URETERAL LIGATION

By H. C. JACKSON

² *Journal of Biological Chemistry*, xi, p. 61, 1912

STUDIES IN NUTRITION

V THE UTILIZATION OF THE PROTEINS OF COTTON SEED

BY LAFAYETTE B MENDEL AND MORRIS S FINE

(From the Sheffield Laboratory of Physiological Chemistry, Yale University,
New Haven, Connecticut)

(Received for publication, September 25, 1911)

The influence of cotton-seed on the well-being of cattle has been extensively investigated in this country, the protein of this material being 88 per cent¹ utilized by steers or sheep. It was of interest to learn to what extent this substance was utilized by dogs, the alimentary canal of which more closely resembles the human digestive tract. Such experiments are of special import, inasmuch as cotton-seed flour bids fair to become an important article in the human dietary. As far as we are aware, an investigation of this nature is not on record.²

EXPERIMENTAL PART

Product Employed

The cotton-seed³ flour of these experiments was a deep yellow impalpable powder, containing 7.4 per cent nitrogen. Fraps⁴ found similar samples to have 4.0 to 6.5 per cent crude fiber. Cotton-seed flour contains some pentosans but no starch.⁵

¹ Cf. Fraps, Texas Agricultural Experiment Station, Bull. 128, 1910.

² Correspondence with Dr. C. F. Langworthy and Dr. Marion Dorsett, of the United States Department of Agriculture, also fail to reveal any literature on this subject.

³ Obtained from the Southern Cotton Oil Company, Charlotte, N. C.

⁴ Fraps, *loc. cit.*

⁵ Fraps, *loc. cit.*

Metabolism Experiments

In Table 1 are recorded three experiments on the utilization of cotton-seed flour. The usual method of procedure⁶ prevailed. The daily supply of cotton-seed contained 2 to 3 grams of crude fiber. The cotton-seed feces of dogs 5 and 6 were hydrolyzed according to the method outlined in a previous paper,⁷ and yielded a daily average of respectively 5 and 3.5 grams of hemicelluloses. The diets of these two dogs, therefore, included 7.5 and 6 grams of indigestible non-nitrogenous substances. This, however, cannot account for the *manifestly poor utilization of the cotton-seed nitrogen*. The coefficients of 67 to 75 per cent for cotton-seed contrast

TABLE 1

Cotton-seed Flour

	Dog 5 PERIOD XIV (4 days) Cotton-seed Feed- ing	Dog 6 PERIOD XX (4 days) Cotton-seed Feed- ing	Dog 7 PERIOD IX (3 days) Cotton-seed Feed- ing
	grams	grams	grams
Composition of daily diet	Cotton-seed	Cotton seed	Cotton-seed
	Flour 45	Flour 45	Flour 45
	Sugar 25	Sugar 25	Sugar 20
	Lard 20	Lard 20	Lard 25
	Water 225	Water 225	Agar 3
			Bone Ash 7
			Water 175
	Estimated calories 410	Estimated calories 410	Estimated calories 440
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	2.61	2.61	2.55
Total nitrogen, gm	3.51	3.70	3.45
Nitrogen in food, gm	3.32	3.32	3.59
Nitrogen balance, gm	-0.20	-0.38	+0.14
<i>Feces</i>			
Weight air dry, gm	23.5	23.9	31.7
Nitrogen, gm	0.91	1.09	0.90
Nitrogen, per cent	3.87	4.57	2.84
Nitrogen utilization, per cent	72.6	67.2	74.9

⁶ Cf. Mendel and Fine. *This Journal*, x, p. 303, 1911.⁷ Cf. Mendel and Fine. *Ibid.*, x, p. 339, 1911.

strikingly with those of 88 to 93 per cent for meat diets containing comparable or greater amounts of such indigestible materials (See Table 2) There is of course the possibility that the cotton-seed flour employed in this study contained some constituent⁸ which either inhibited secretion or promoted premature evacuation—conditions which would result in poor utilization

TABLE 2

*Utilization with Reference to Indigestible Materials in the Diet **
Daily Averages

DOG	PERIOD	DAYS	NATURE OF INGESTA	FIBER CONTAINED IN EXPERIMENTAL DIET, ON ADDED TO THE MEAT	TOTAL VOLUME OF IN- DIGESTIBLE MATERIAL IN FOOD	NITROGEN INTAKE	NITROGEN UTILIZATION	AVERAGE NITROGEN UTILIZATION
				grams	grams	grams	per cent	per cent
5	XV	4	Cotton seed	3	8†	3.3	72.6	71.6
6	XV	4		3	6†	3.3	67.2	
7	IX	3		3	14†	3.6	75.0	
5	XVIII	4	Meat	6	6	3.3	90.5	91.0
6	XV	4		6	6	3.3	89.2	
7	XVIII	4		6	6	3.3	93.3	
5	XV	4	Meat	6	13	3.3	91.6	89.2
6	XVI	4	Bone ash, 5 grams	6	13	3.3	87.7	
7	XV	4	Agar, 2 grams	6	13	3.3	88.3	

* This problem will be treated in detail in a subsequent paper of this series

† Including respectively about 5 grams and 3 grams hemicelluloses which escaped digestion

‡ Including approximately 4 grams indigestible hemicelluloses (exclusive of agar) i.e. the average of 5 grams and 3 grams. An actual determination of the hemicelluloses of the feces of this experiment was not made

⁸ Cf Crawford *Journ of Pharm and Exper Ther*, 1, No 5, p 519, 1910

STUDIES IN NUTRITION

VI THE UTILIZATION OF THE PROTEINS OF EXTRACTIVE-FREE MEAT POWDER, AND THE ORIGIN OF FECAL NITROGEN

BY LAFAYETTE B MENDEL AND MORRIS S FINE

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(Received for publication, September 25, 1911)

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THE UTILIZATION OF EXTRACTIVE-FREE MEAT POWDER

EARLIER STUDIES

Forster was the first to conduct an investigation with this material. His immediate problem was the question of salt metabolism, but incidentally we note that the nitrogen was 91 to 96 per cent available. During the past twenty years, considerable attention has been paid to the comparative utilization of fresh meat and

dried meat preparations, for example, "soston," "somatose," "tropon," and the meat residues from meat extract factories. Passing over the literature previous to 1901, we may dwell briefly upon the results obtained by Prausnitz, which are in general accord with those of the earlier workers. The average coefficient of digestibility of dried meat was 90 per cent against a coefficient of 93 per cent for fresh meat. Moreover the nitrogen concentration of the dried-meat-feces was 1.35 to 1.76 per cent higher than the fresh meat feces. These facts make it probable that a portion of the dried meat had escaped absorption. Prausnitz also showed that dried meat was less readily digested in artificial gastric juice than fresh meat. He accounted for these phenomena on the assumption that a not inappreciable length of time elapses before the dried meat particles are sufficiently "hydrated" to permit the digestive enzymes to operate. Max Voit found similar although less striking differences.

Considerable work has also been accomplished with dried blood preparations, but a consideration of these investigations would lead us too far afield.

EXPERIMENTAL PART

Product Employed

The meat residue¹ employed in the present studies was a light brown impalpable powder, containing 13.2 per cent of nitrogen, 8.9 per cent of ether extract, 2.5 per cent of ash, and 7.0 per cent of moisture.

Metabolism Experiments

Tables 1-3. During these experiments, the methods described in a previous paper² were followed. *The utilization of the nitrogen of meat powder is distinctly, although slightly lower than that of fresh meat. The relatively high nitrogen concentration of the meat powder feces is indicative of a loss of this material through the excrement.* These points are concisely presented in the accompanying brief tabular summary.

¹ Obtained from Armour and Company.

² Mendel and Fine. *This Journal*, v, p. 303, 1911.

Summary of the Data on Nitrogen Utilization (see Tables 1-3)

DOG	MEAT POWDER		FRESH MEAT (AVERAGES)	
	Nitrogen utilization	Nitrogen in feces	Nitrogen utilization	Nitrogen in feces
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	91.3	2.98	94.0	1.94
4	89.3	3.81	94.5	2.04
4	91.0	3.87	93.7	2.36

TABLE 1

Extract-free Meat Powder

SUBJECT DOG 1 Weight at beginning 14.6 kg Weight at end 14.6 kg		PERIOD V (4 days) Meat Feeding	PERIOD VI* (5 days) Meat Powder Feeding	PERIOD VII (4 days) Meat Feeding
Composition of daily diet		<i>grams</i>	<i>grams</i>	<i>grams</i>
		Meat 300	Meat Powder 80	Meat 300
		Lard 60	Lard 60	Lard 60
		Agar 5	Agar 5	Agar 5
		Bone ash 15	Bone Ash 15	Bone Ash 15
		Water 300	Water 500	Water 300
		Estimated calories 1070	Estimated calories 860	Estimated calories 1070
<i>Nitrogen output</i>		Daily Averages	Daily Averages	Daily Averages
Urine nitrogen, gm		8.81	8.76	8.47
Total nitrogen, gm		9.38	9.68	9.15
Nitrogen in food, gm		10.44	10.53	10.46
Nitrogen balance, gm		+1.06	+0.85	+1.31
<i>Feces</i>				
Weight air dry		29.5	31.0	35.2
Nitrogen, gm		0.57	0.92	0.68
Nitrogen, per cent		1.95	2.98	1.92
Nitrogen utilization, per cent		94.5	91.3	93.5

* Food almost entirely forced

TABLE 2

Extract-free Meat Powder

SUBJECT DOG 4 Weight at beginning 4.9 kg Weight at end 5.1 kg	PERIOD V (4 days) Meat Feeding	PERIOD VI (5 days) Meat Powder Feeding	PERIOD VII (4 days) Meat Feeding
	<i>grams</i>	<i>grams</i>	<i>grams</i>
Composition of daily diet	Meat 150	Meat 150	Meat 150
	Sugar 25	Powder 39	Sugar 25
	Starch 5	Sugar 25	Starch 5
	Lard 20	Starch 5	Lard 20
	Bone Ash 10	Lard 25	Starch 5
	Water 200	Agar 8	Lard 20
		"Salts" 4	Agar 8
		Water 260	"Salts" 4
	<i>Estimated</i>	<i>Estimated</i>	<i>Estimated</i>
	calories 570	calories 510	calories 570
	Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>			
Urine nitrogen, gm	4.23	3.95	4.37
Total nitrogen, gm	4.50	4.50	4.70
Nitrogen in food, gm	5.40	5.13	5.40
Nitrogen balance, gm	+0.90	+0.63	+0.70
<i>Feces</i>			
Weight air dry, gm	15.0	14.4	14.0
Nitrogen, gm	0.27	0.55	0.32
Nitrogen, per cent	1.79	3.81	2.29
Nitrogen utilization, per cent	95.0	89.3	94.1

TABLE 3

Extract-free Meat Powder

SUBJECT DOG 4 Weight at beginning 5.1 kg Weight at end 5.2 kg		PERIOD VI (5 days) Meat Feeding	PERIOD VII (5 days) Meat Powder Feeding	PERIOD VIII (4 days) Meat Feeding
		grams	grams	grams
Composition of daily diet	Meat	150	Meat	150
			Powder	40
	Sugar	25	Sugar	25
	Starch	5	Starch	5
	Lard	20	Lard	25
	Agar	8	Agar	8
	"Salts"	4	"Salts"	4
	Water	200	Water	300
		Estimated calories 570	Estimated calories 510	Estimated calories 570
		Daily Averages	Daily Averages	Daily Averages
<i>Nitrogen output</i>				
Urine nitrogen, gm		4 28	4 51	4 46
Total nitrogen, gm		4 62	4 98	4 77
Nitrogen in food, gm		5 20	5 26	5 22
Nitrogen balance, gm		+0 58	+0 28	+0 45
<i>Feces</i>				
Weight air dry, gm		12 4	12 3	16 0
Nitrogen, gm		0 34	0 47	0 31
Nitrogen, per cent		2 77	3 87	1 96
Nitrogen utilization, per cent		93 4	91 0	94 0

ON THE ORIGIN OF FECAL NITROGEN

In previous papers³ of this series we have followed the current custom of basing the data for nitrogen utilization upon the relation of the nitrogen appearing in the excrement to that of the ingesta. This procedure would be strictly correct only in case the fecal nitrogen consisted entirely of food residues. As a matter of fact, there is abundance of evidence in the literature to demonstrate that fecal nitrogen in great part emanates from "metabolic products."⁴ Obviously an adequate understanding of the source of fecal nitrogen and the conditions influencing its excretion is essential for the proper interpretation of experiments on nitrogen utilization. In the earlier papers referred to we have at times pointed out that an apparently poor utilization was probably induced by the indigestible matter—cellulose, hemicellulose—inherent in the experimental material. The influence of such materials upon utilization has not always been fully appreciated. Rubner, and later Wicke, did indeed call attention to the unfavorable effect of cellulose upon the utilization of bread nitrogen, but in these cases it is difficult to decide in what measure the insufficiently ruptured cells are responsible for the low coefficients of digestibility, and to what extent the latter is to be attributed to the cellulose *per se*. This question is not satisfactorily answered by the poor utilization of meat obtained by Hoffmann when coarsely cut straw was added to the diet. Such coarse particles probably unduly irritated the digestive tract, resulting in increased secretion and peristalsis. Lothrop demonstrated an increased elimination of fecal nitrogen when bone ash was added to the diet.

In the present paper the nitrogen of the excrement under a variety of conditions is discussed briefly from the historical aspect,⁵ data purporting to show to what extent indigestible non-nitrogenous substances may influence the amount and character of the feces are presented, and a plan of experimentation is proposed,

³ See footnotes 20-24, pp. 23 and 24.

⁴ By this term is understood intestinal secretions, cast off cells, bacteria, etc. For a consideration of the important rôle of bacteria in this respect and the literature related thereto, see MacNeal, Latzer and Kerr *Journ of Infect Dis.*, vi, p. 123, 1909.

⁵ For a more detailed review reference is made to Tsuboi (see bibliography).

with which it seems possible to approximately determine to what degree the nitrogen excreted in the feces is derived from undigested or indigestible nitrogenous constituents of the ingesta. Were this known, the term "utilization" would be eminently appropriate.

EARLIER STUDIES

Feces in Starvation

Man The accompanying table presents oft quoted data⁶ obtained from the professional fasters, Cetti and Breithaupt, and from certain patients

Daily Nitrogen Excreted through the Feces in Starvation

	gram
Cetti	0 32
Breithaupt	0 12
Patient (stenosis of oesophagus)	0 45
Neurasthenic	0 22
Neurasthenic	0 17
Average	0 26

Dogs Bidder and Schmidt, and Voit early observed that during starvation black pitch-like feces were obtained from dogs. The latter obtained daily 2 grams of feces (= 0 15 gram of nitrogen) from a dog of 30 kilos. The studies of Müller offer further illustrative data.

Daily Feces Obtained from Starving Dogs (Müller, 1884)

BODY WEIGHT	FECES WEIGHT DRY	FECAL NITROGEN		FECAL NITROGEN PER KILO BODY WEIGHT
kilo	grams	per cent	grams	gram
43	4 8	5 0	0 24	0 0056
30	2 4	8 0	0 19	0 0063
30	1 4	8 0	0 11	0 0037
23	2 8	5 3	0 15	0 0065
7	0 7	7 5	0 05	0 0071
Average				0 0058

⁶ Taken from Schmidt and Strasburger (see bibliography), p 115

Benedict has pointed out that the amount of feces formed during starvation is probably much smaller than is indicated by earlier studies. Fasting feces are in great part derived from retained fecal matter, resulting from the food immediately preceding the period of inanition. This is owing to diminished peristalsis consequent upon the withdrawal of food.

With Nitrogen-Free Diets

The accompanying table embodies results obtained by Rieder

Nitrogen Eliminated through Feces on Nitrogen-free Diet (Rieder)

SUBJECT	FECES WEIGHT DRY	FECAL NITROGEN		FOOD
	grams	per cent	gram	
Man	13.4	4.08	0.54	485 grams cakes of starch, sugar and fat
Man	15.4	5.69	0.87	159 grams cakes of starch, sugar and fat
Man	13.4	5.85	0.78	147 grams cakes of starch, sugar and fat
Dog	3.0	3.67	0.11	70 grams starch
Dog	6.0	3.85	0.22	140 grams starch

Rubner (1879) reported similar results. Tsuboi fed dogs for periods of six to nine days on cakes made of starch, fat and sugar, and obtained data, which are in accord with the above.

Nitrogen Eliminated through Feces on Nitrogen-free Diet (Tsuboi)

FECES WEIGHT DRY	FECAL NITROGEN		FOOD		
			Starch	Sugar	Fat
grams	per cent	gram	grams	grams	grams
2.6	5.1	0.14	0	0	0
5.8	4.1	0.24	70	12	50
12.9	4.4	0.57	200	25	80

There can of course be no question as to the source of fecal nitrogen in the above experiments.

With Meat Diets

The most interesting work bearing upon the nitrogen of the feces obtained with meat diets and the relation of the amount of meat ingested to the nitrogen thus eliminated was contributed by Müller

Influence of Meat Diet on Fecal Nitrogen in Dogs (30-35 kilos) (Müller)

MEAT	FECES WEIGHT DRY	FECAL NITROGEN		NITROGEN UTILIZED
grams	grams	per cent	gram	per cent
0	2 0	7 96	0 15	
500	5 1	6 50	0 30	98 2
1000	9 2	6 50	0 55	98 4
1500	10 2	6 50	0 67	98 7
1800	10 3	6 50	0 70	98 9
2000	11 1	6 50	0 80	98 8
2500	15 4	6 50	1 00	98 8

It is clear from this summary that the nitrogen of the feces does not increase in proportion to the amount of meat eaten

That the fecal nitrogen incident to a meat diet is essentially of metabolic origin,⁷ is very convincingly brought out by Fritz Voit. After a loop of the intestine had been isolated, a dog was fed with meat. It was found that the contents of the loop resembled the feces in appearance and nitrogen content. Moreover when calculated to unit surface the absolute amount of dry substance in the loop compared favorably with that of the feces. Equally significant is the recent study of Mosenthal, who also worked with isolated intestinal loops. This author estimated that the succus entericus contained nitrogen equivalent to 35 per cent of the nitrogen ingested, and 300 to 400 per cent of the nitrogen of the feces. Nitrogen equivalent to at least 25 per cent of that of the intake must therefore have been reabsorbed.

From the foregoing there can be no doubt that the feces resulting from a thoroughly digestible food such as meat are almost solely of "metabolic origin." Prausnitz has attempted to give this more widespread application

⁷ By an ingenious microscopical method, Kermauner (see bibliography) showed that in man but one per cent or less of the ingested meat reappeared in the feces

Composition of Feces on Various Diets (Prausnitz)

NUMBER	PERSON	MAIN FOOD	FECES—DRY		
			Nitrogen	Ether Extract	Ash
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	H	Rice	8 83	12 4	15 4
2	H	Meat	8 75	16 0	14 7
3	M	Rice	8 37	18 2	11 0
4	M	Meat	9 16	16 0	12 2
5	W P	Rice	8 59	15 9	12 6
6	W P	Meat	8 48	17 5	13 1
7	J Pa	Rice	8 25		14 5
8	J Pa	Meat	8 16		15 2
9	F P ₁	Rice	8 70		16 1
10	F P ₁	Meat	9 05		15 1
11	d Cl (vegetarian)	Rice	8 78	18 6	12 0
		Average	8 65	16 4	13 8
12	M	Mixed diet	6 76	25 3	12 0
13	H	Mixed diet	6 63	25 8	14 9
14	H	Mixed diet	6 07	30 1	15 0

The excreta from the above diets (Nos 1 to 11) contained no starch, and the composition of the feces did not alter materially as the character of the food changed. Such feces Prausnitz considered "normal feces." When, however, the food contains material of a less digestible nature, the composition may change. Where this indigestible material is cellulose the nitrogen content of the feces is lowered (Nos 12 to 14), if a nitrogenous substance, the nitrogen content might be expected to be raised.

Schierbeck recognizes three types of individuals (1) those that consistently have feces with low nitrogen concentration (about 4 per cent) whatever the nature of the diet may be, (2) those that under these conditions have feces of high nitrogen percentage (6-7 per cent), and (3) those in whom coarse food yields feces of low nitrogen percentage, and readily absorbed material produces feces with nitrogen concentration as high as 8 per cent.

We are inclined to agree with Benedict that during starvation the formation of feces is reduced to a practically negligible quantity. When a material such as meat is eaten whose protein utilization, estimated according to the usual custom, is at least 95

per cent, the resulting feces are for the most part of metabolic origin. *It has been shown that the feces from such a diet represent a very small portion of the originally secreted intestinal juice, the latter having been absorbed in great part before reaching the rectum. Obviously the degree to which this secretion is reabsorbed will depend upon the rate of peristalsis, which in turn is influenced by the mass and character of material in the intestine. Hence, if to a meat diet an indigestible or less digestible material is added, thus stimulating peristalsis, more metabolic products¹ must escape reabsorption. If we deal with a non-nitrogenous material, e g, agar, bone ash or crude fiber, the percentage nitrogen of the feces will of course be lower. If the comparatively indigestible material is highly nitrogenous like protein, the nitrogen concentration will be higher, and if both types of indigestible materials are present, the percentage of nitrogen may be indistinguishable from that found in meat-feces. Illustrative data follow.*

EXPERIMENTAL PART

The conduct of these experiments did not differ essentially from that of trials described in previous papers. The quantities of meat and indigestible non-nitrogenous materials can be learned from the tables, the amounts of water, sugar and lard approximated those employed in previous experiments.

The influence of indigestible non-nitrogenous materials upon the nitrogen statistics of the feces is illustrated in Tables 4 and 5. In Table 4 the contrast is made between feces resulting from meat and feces accruing from an identical diet to which 3 grams of agar plus 7 grams of bone ash had been added daily. In Table 5 a similar contrast is drawn between meat- and meat-crude-fiber feces. The data are briefly summarized in Table 6. The increase in absolute fecal nitrogen due to the addition of indigestible materials to the diet is manifest, although the nitrogen intake did not vary. Thus the fecal nitrogen of (1) is increased 60 per cent by the addition of 10 grams of indigestible non-nitrogenous substances, and that of (3) is augmented 133, 133, and 192 per cent²

¹ Possibly also food residues and products of digestion

² Too great a quantitative significance should not be placed upon these figures, as an accurate isolation of pure meat-feces is almost impossible even when special precautions are taken

TABLE 4

*Influence of Agar + Bone Ash upon the Feces Resulting from a Meat Diet
Daily Averages*

NUMBER	DOG	DAYS	PERIOD	NATURE OF INGESTA	FECES			NITROGEN UTILIZATION
					Weight Air Dry	Nitrogen	Nitrogen	
					grams	gram	per cent	per cent
1	5	4	XX	{ Meat, sugar, lard = 46 to 49 gm nitrogen }	4 5	0 22	4 95	95 2
2	5	5	XXVIII		3 4	0 16	4 62	96 6
3	5	5	I	{ As above + { Agar 3 gm Bone Ash 7 gm }	13 2	0 29	2 22	94 0
4	5	4	III		14 5	0 36	2 48	92 7
5	5	4	IV		15 5	0 40	2 60	91 8
6	5	5	VIII		15 0	0 35	2 32	92 7
				<i>Average of 1 and 2</i>	4 0	0 19	4 78	95 9
				<i>Average of 3 to 6</i>	14 5	0 37	2 40	92 4
7	6	4	XXI	{ Meat, etc , as for Dog 5 }	3 5	0 22	0 34	95 2
8	6	5	XXIX		4 4	0 24	5 42	94 9
9	6	4	I	{ Meat, etc , with indigestible materials, as for Dog 5 }	12 2	0 28	2 28	94 3
10	6	5	IV		13 6	0 36	2 64	92 7
11	6	4	VI		14 5	0 35	2 42	92 9
12	6	5	VIII		15 0	0 39	2 59	92 0
				<i>Average of 7 and 8</i>	4 0	0 23	5 88	95 0
				<i>Average of 9 to 12</i>	13 8	0 34	2 48	93 0
13	7	4	XX	{ Meat, etc , as for Dog 5 }	3 2	0 19	5 93	95 8
14	7	5	XXVIII		3 8	0 15	3 82	96 9
15	7	4	I	{ Meat, etc , with indigestible materials, as for Dog 5 }	12 5	0 28	2 26	91 4
16	7	5	III		12 8	0 23	1 81	93 0
17	7	3	V		12 7	0 26	2 04	92 1
18	7	5	VII		12 8	0 24	1 86	92 6
				<i>Average of 13 and 14</i>	3 5	0 17	4 88	96 5
				<i>Average of 15 to 18</i>	12 7	0 25	1 99	92 5

TABLE 5

Influence of Crude Fiber upon the Feces Resulting from a Meat Diet
Daily Averages

NUMBER	DOG	DAYS	PERIOD	NATURE OF INGESTA	FECES			NITROGEN UTILIZATION
					Weight Air Dry	Nitrogen	Nitrogen	
					grams	gram	per cent	per cent
1	5	4	xvi	Meat, etc, = 33 gm nitrogen	0 41	0 021	5 32	99 4†
2	5	4	xviii	As above + 6 gm crude fiber*	10 1	0 30	2 97	90 5
3	6	4	xvii	Meat, etc, as for Dog 5	1 9	0 13	7 03	96 0
4	6	4	xix	Meat, etc, + 6 gm crude fiber as for Dog 5	10 0	0 34	3 52	89 2
5	7	4	xvi	Meat, etc, as for Dog 5	1 5	0 10	7 06	96 8
6	7	4	xviii	Meat, etc, + 6 gm crude fiber as for Dog 5	8 5	0 21	2 51	93 3
				Average of 1, 3, 5	1 7	0 12	6 47	96 4
				Average of 2, 4, 6	9 5	0 28	3 00	91 0
7	5	4	xii	Meat, etc, + 2 gm agar + 5 gm bone ash	11 0	0 28	2 59	91 8
8	5	4	xiv	The same	11 5	0 26	2 27	92 1
9	5	4	xv	The same + 6 gm filter paper	17 7	0 27	1 55	91 6
10	6	4	xiii	Meat, etc, + 2 gm agar + 5 gm bone ash	10 5	0 35	3 37	89 8
11	6	4	xv	The same	10 0	0 31	3 07	90 6
12	6	4	xvi	The same + 6 gm filter paper	18 0	0 40	2 23	87 7
13	7	4	xiii	Meat, etc, + 2 gm agar + 5 gm bone ash	8 5	0 23	2 74	93 3
14	7	4	xiv	The same	9 5	0 26	2 70	92 2
15	7	4	xv	The same + 6 gm filter paper	18 0	0 38	2 14	88 3
				Average of 7, 8, 10, 11, 13, 14	10 2	0 28	2 79	91 6
				Average of 9, 12, 15	17 9	0 35	1 97	89 2

* Newspaper (0.1 per cent nitrogen) was thoroughly disintegrated under water

† These values are abnormally low owing to poor separation of feces of successive periods. They are not included in the averages.

** Omitted from the averages

TABLE 6

The Influence of Indigestible Non-Nitrogenous Materials upon the Nitrogen Statistics of Meat-Feces (Summary of Tables 4 and 5) Daily Averages

REFERENCE NUMBER	NUMBER OF EXPERIMENTS AVERAGED	NITROGEN INTAKE	TOTAL VOLUME INDIGESTIBLE NON-NITROGENOUS MATERIAL ADDED TO THE MEAT	FECES			NITROGEN UTILIZATION
				Weight (Air Dry)	Nitrogen	Nitrogen	
		grams	grams	grams	gram	per cent	per cent
1	6*	4 6	0	3 8	0 20	5 2	95 7
2	12**	4 6	10	13 7	0 32	2 3	92 7
3	3†	3 3	0	1 7	0 12	6 5	96 4
4	3‡	3 3	6	9 5	0 28	3 0	91 0
5	6§	3 3	7	10 2	0 28	2 8	91 6
6	3	3 3	13	17 9	0 35	2 0	89 2

* Cf Table 4, Nos 1, 2 7 8, 13, 14

** Cf Table 4, Nos 3 to 6 9 to 12 15 to 18

† Cf Table 5, Nos 1 3, 5

‡ Cf Table 5, Nos 2 4, 6

§ Cf Table 5, Nos 7, 8, 10 11, 13, 14

|| Cf Table 5, Nos 9, 12, 15

by the addition to the diet of 6, 7, and 13 grams respectively of such materials. The low nitrogen concentration of the feces of (2), (4), (5), and (6) is characteristic of diets of thoroughly utilized materials including much indigestible non-nitrogenous matter. The nitrogen concentration, however, is not sufficiently diminished to compensate for the increased volume of feces—hence the above increment in absolute fecal nitrogen and the correspondingly lowered coefficients of digestion.

Illustrations of the influence of poorly utilized highly nitrogenous matter upon the nitrogen statistics of the feces are especially conspicuous in certain data already published¹⁰ and which are reproduced in Table 7. The nitrogen concentration of the phaseolin-feces is 6.1 per cent against 2.3 per cent for that of feces resulting from a meat diet fed under conditions identical with those attending the phaseolin feeding. A similar though less striking example

¹⁰ Mendel and Fine. *This Journal* v, p 433, 1912. Table 24 (phaseolin), Table 25 (pea globulin), Tables 10–11 (soy bean).

is offered in the case of the pea globulin experiment Nos 5 to 8 of this table disclose how closely the *nitrogen concentration of feces accruing from diets containing both poorly utilized highly nitrogenous materials and indigestible non-nitrogenous materials may simulate the corresponding value for meat feces*

TABLE 7

The Influence of Poorly Utilized Highly Nitrogenous Materials upon the Nitrogen Statistics of the Feces Daily Averages

REPORT NUMBER	NATURE OF INGESTA	NITROGEN INTAKE	FECES			NITROGEN UTILIZATION
			Weight (Air Dry)	Nitrogen	Nitrogen	
		grams	grams	grams	per cent	per cent
1	Phaseolin	5.2	20.0	1.21	6.1	76.9
2	Meat*	5.2	10.9	0.26	2.3	95.0
3	Pea Globulin	4.8	15.6	0.56	3.6	88.3
4	Meat†	4.8	14.7	0.37	2.5	92.4
5	Soy Bean	4.6	15.9	0.57	3.6	87.6
6	Meat	4.6	3.8	0.15	3.8	96.9
7	Soy Bean	3.3	14.0	0.65	4.7	80.2
8	Meat	3.3	0.4‡	0.02‡	5.3	99.4

Average of fore and after periods

* Average of fore and after periods

† See second note to Table 5 this paper

Obviously the fecal nitrogen concentration *by itself* is not a safe criterion¹¹ by which to judge the digestibility of a material. The nitrogen of the voluminous meat-cellulose-feces may be almost entirely of metabolic origin and yet be present in relatively low concentration, whereas a soy bean diet may yield feces composed in great part of highly nitrogenous undigested food residues, the nitrogen concentration,¹² however, being comparable to that of meat-feces.

¹¹ Tsuboi (see bibliography), p. 80 likewise believes that one should be conservative in drawing conclusions from this one factor.

¹² Tsuboi (*loc cit*, p. 81), has made a similar statement. He points out that in Rubner's studies, peas were poorly utilized (72 per cent) and yet the nitrogen concentration of the feces was 7.3 per cent, thus according closely with that of 6.9 per cent for the nitrogen concentration of feces from meat which was 97 per cent utilized.

Benedict has called attention to the difficulty encountered in satisfactorily isolating feces accruing from a particular diet, owing to the lagging behind of fecal material from the preceding diet. Our own experience testifies to this difficulty. It was especially pronounced where the experimental, preceding and succeeding

TABLE 8
Influence of Thorough Evacuation upon Nitrogen Statistics of Feces
Daily Averages

NUMBER	DOG	DAYS	PERIOD	NATURE OF INGESTA	FECES			NITROGEN UTILIZATION
					Weight Air Dry	Nitrogen	Nitrogen	
					grams	gram	per cent	per cent
1	5	4	xxiii	Meat, etc , + 10 gm Agar (= 4.6 gm Nitrogen	15.7	0.52	3.33	88.6
2	5	3	xxiv	Meat, etc , + 10 gm Bone Ash	13.8	0.26	1.90	94.3
3	5	4	xxv	Meat, etc , + 10 gm Agar	13.5	0.40	2.95	91.4
4	6	4	xxiv	Meat, etc , + 10 gm Agar	15.5	0.53	3.42	88.5
5	6	3	xxv	Meat, etc , + 10 gm Bone Ash	15.2	0.32	2.12	93.1
6	6	4	xxvi	Meat, etc , + 10 gm Agar	12.8	0.37	2.92	92.0
7	7	4	xxiii	Meat, etc , + 10 gm Agar	14.8	0.45	3.02	90.3
8	7	3	xxiv	Meat, etc , + 10 gm Bone Ash	14.0	0.28	1.99	94.0
9	7	4	xxv	Meat, etc , + 10 gm Agar	11.9	0.32	2.70	93.1
Average of 1, 4, 7					15.3	0.50	3.26	89.1
Average of 2, 5, 8					14.3	0.29	2.00	93.8
Average of 3, 6, 9					12.7	0.36	2.86	92.2

diets were all composed of thoroughly digested materials and the resulting feces were not adequate stimuli to peristalsis. This difficulty was obviated in a measure when the experimental period was preceded and succeeded by a 2-3 day period of a meat diet including 10 grams of bone ash daily.

This lag and the effect of previous thorough evacuation upon utilization is illustrated in Table 8. The first period for each dog (Nos 1, 4, 7) was preceded by a period of wheat gluten, which is very well utilized.¹³ After thorough evacuation, it is clear (Nos 3, 6, 9) that the apparent utilization is considerably improved.

Estimation of "Metabolic" Products in the Feces

Investigators have sought a method whereby the prominent part taken by alimentary waste products in the formation of feces could be determined with some degree of accuracy. This would enable one to estimate what proportion of the feces is due to undigested food residues. Processes have been proposed which involve treating the feces with pepsin-HCl or dilute alkali. Data thus obtained are of doubtful value. Equally unsatisfactory are those procedures which involve subtracting from the experimental feces the equivalent of fecal material obtained during starvation or on a thoroughly digested non-nitrogenous diet. The plan generally followed in the present work, namely the comparison of experimental feces with feces obtained from a control meat diet is likewise not always free from objection. None of the above methods take into account the influence of undigested masses upon the degree of reabsorption of the intestinal juice. We propose the following plan¹⁴ which seems to avoid most of the above shortcomings.

- 1 Determine the volume and nitrogen of feces resulting from the material under investigation.
- 2 Determine the fecal nitrogen resulting from a nitrogen-free diet to which has been added an amount of indigestible non-

¹³ Cf Mendel and Fine. *This Journal*, x, p 324, 1911.

¹⁴ Tsuboi has applied a similar principle to certain results reported by Rubner. The nitrogen eliminated on a starch diet was subtracted from that excreted in feces of comparable volume resulting from diets of wheat bread and maccaroni. The food nitrogen actually escaping utilization could thus be computed.

nitrogenous matter¹⁵ that will yield approximately the same volume of feces as was obtained in (1)

3 Subtract the fecal nitrogen of (2) from that of (1) This excess of nitrogen is presumably due to undigested or unabsorbed nitrogenous matter of the food material

An experiment with a nitrogen-free diet including indigestible non-nitrogenous matter follows

A 6 kilo bitch was fed for four days on a mixture of 35 grams of sugar, 45 grams of lard, 200 grams of water and 10 grams of agar On this diet 13.2 grams of feces with a nitrogen concentration of 2.44 per cent were obtained daily There were thus eliminated through the feces 0.32 gram of nitrogen daily, which was obviously of metabolic origin This result makes it probable that the feces from *meat diets*, containing similar amounts of indigestible non-nitrogenous matter, (see Table 6) are likewise made up entirely of alimentary waste—proof in itself that meat nitrogen is 100 per cent utilized

From the single experiment above reported and from Table 6, No. 6, we may conclude that a thoroughly digested material may yield 13.2 to 17.9 grams of feces and yet the nitrogen (0.32–0.35 grams) thus eliminated will be of “metabolic” origin Hence in feces of comparable volumes¹⁶ all nitrogen in excess of 0.32–0.35 gram may be attributed to the nitrogen of the food This principle is applied in Table 9

“Utilization,” as the term is employed in the last column of this table, exactly expresses our meaning The actual utilization of soy bean nitrogen¹⁷ is 90.3–92.8 per cent and that for the crude bean protein is 91.8 per cent If anything the latter value is low, as 24.6 grams of meat-feces would probably contain more than 0.35 gram of nitrogen

¹⁵ The choice of indigestible adjuvant is a matter of some moment, as these materials may vary in their ability to stimulate peristalsis

¹⁶ This of course applies only for dogs of approximately the same weight (5 to 7 kilos) as those in these experiments

¹⁷ Soy bean is reported (Wolff-Lehmann *Landw. Fütterungslehre*, cited by Schulze und Castoro *Zeitschr. f. physiol. Chem.*, xli, p. 455, 1904) as having 10 per cent of its nitrogen present as non-protein The latter may be more thoroughly utilized than the protein constituents, and thus the utilization calculated for the total nitrogen intake would be greater than is actually the case for the soy bean protein Excepting the soy bean and cotton-seed flours, the preparation of the materials employed in this series of studies renders contamination with nitrogenous non-protein matter unlikely

The Utilization of the Vegetable Proteins

About the thorough utilization of the proteins of wheat¹⁸ there is no question. The probability that those of barley¹⁹ and corn²⁰ are equally available was pointed out in previous papers of this series. With regard to the legume proteins²¹ we must for the present conclude that the presence of indigestible non-nitrogenous materials cannot entirely account for their low coefficients of diges-

TABLE 9

Utilization as Estimated from the Portion of Fecal Nitrogen Derived from Food Residues Daily Averages

NUMBER OF FX PERIMENTS AVERAGED	NATURE OF INGESTA	NITROGEN INTAKE	FECES		NITROGEN FROM UNDIGESTED FOOD	NITROGEN UTILIZATION	
			Weight (Air Dry)	Nitrogen		As Ordinarily Estimated	Actual Utilization
		gram	grams	gram	gram	per cent	per cent
1	Nitrogen-free diet including 10 gm agar	0 0	13 2	0 32	0 00		
3	Meat diet*	3 3	17 9	0 35	0 00	89 2	100 0
6	Soy bean†	4 6	17 2	0 68	0 33	85 3	92 8
3	Soy bean‡	3 3	13 0	0 64	0 32	81 1	90 3
1	Crude bean protein§	3 4	24 6	0 63	0 28	81 5	91 8

Cf Table 6 No 6 this paper

† Cf Mendel and Fine *This Journal* x p 433 1912 Tables 5-10

‡ Cf Mendel and Fine *loc cit* Tables 11 to 13

§ Cf Mendel and Fine *loc cit* Table 21

tibility. These proteins appear to be less readily affected by the digestive processes than those of barley or corn. This resistance is even more pronounced in the case of the cotton-seed protein²². Nevertheless, future research with the isolated proteins may modify our opinion with regard to these two last classes of materials.

The lack of animal extractives in vegetable materials has at times been thought to be the cause of the apparently poor utilization of plant foods in comparison with those of animal origin.

¹⁸ Cf Mendel and Fine *This Journal*, v, p 303, 1911

¹⁹ Cf Mendel and Fine *Ibid* v, p 339, 1911

²⁰ Cf Mendel and Fine *Ibid*, v, p 345, 1911

²¹ Cf Mendel and Fine *Ibid* v, p 433, 1912

Cf Mendel and Fine *Ibid* vi, p 1, 1912

ORIGIN OF FECAL NITROGEN AND CONDITIONS INFLUENCING ITS EXCRETION

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CHEMISTRY OF THE DOG'S SPLEEN¹

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In conjunction with experimental work on the histological and chemical changes in the spleen during autolysis, results of which are to be published later, it was found desirable to analyze spleens and also to study their purine enzymes. The results of these studies are given in this paper.

The dog's spleen has never been analyzed in a complete manner chemically, but has frequently been used for the study of its enzymes and individual elements. The spleen of man and other animals has, however, been studied more fully. The water content of the spleen is given as about 78 per cent,³ 70 per cent to 77 per cent,⁴ Schulz⁵ finding 79.5 per cent in human spleen. 12.5 per cent of the spleen is blood content.⁶ The fat and lipid content, which is probably variable, is given as about 11 per cent to 14 per cent of the dry weight. The phosphorus content of cow's spleen varies with age, the spleen of fetuses being richer in phosphorus (up to 2.4 per cent of the dry weight) while adult spleens have less phosphorus (1.3 to 1.4 per cent of the dry weight).

The sulphur content of the cow's spleen is fairly constant in the different periods of life,⁷ ranging from 1.8 per cent to 2.2 per cent.

¹ This work has been aided by a grant from the Rockefeller Institute for Medical Research.

² A portion of this work was done in the Physiological Laboratory of the University of Illinois.

³ Oppenheimer's *Handbuch der Biochemie*, II, 2, p. 172, 1909.

⁴ S. Fränkel, *Descriptive Biochemie*, Wiesbaden, 1907.

⁵ Schulz, *Pflüger's Archiv*, LV, pp. 555-573, 1893.

⁶ Oppenheimer's *Handbuch der Biochemie*, II, 2, p. 172, 1909.

⁷ *Zeitschr. f. Biol.*, XXX, pp. 400-413, 1895.

of the dry weight Schulz⁸ examined the spleen of a man thirty-nine years old and found a water content of 79.5 per cent and a sulphur content of 0.78 per cent of the dry weight

The iron content is also variable, differing markedly in different periods of life,⁹ and the iron is held mostly in organic molecules¹⁰

The proteins of the spleen have been very little studied, investigation along this line being mainly confined to the study of the nucleoproteins and purines. Mandel and Levene¹¹ hydrolyzed the spleen nucleoprotein and obtained glutamic acid, glycocoll, alanine, aspartic acid, proline, phenylalanine, tyrosine, lysine, arginine, histidine, adenine, guanine, cytosine and thymine

The ammonia content of the organs of the body varies, being greater than normal during hunger, as determined by the Folin method. Grafe¹² examined one spleen, that of a horse and found 9.5 mg of ammonia per 100 grams of spleen. The fresh spleen contains neither albumoses nor peptones. Levene¹³ found as end products of the autolysis of the spleen, alanine, leucine, amino-valerianic acid, aminobutyric acid, and α -pyrrolidine carboxylic acid, phenylalanine, aspartic acid and tyrosine. Adenine and guanine were replaced by hypoxanthine and xanthine, and thymine, cytosine and uracil, which are present in spleen nucleic acid, were found as thymine and uracil after autolysis of the spleen. Jones¹⁴ obtained from autolyzed pig spleen guanine and hypoxanthine, but no adenine, while in place of the thymine and cytosine which is found after hydrolysis, he obtained uracil. Later Jones¹⁵ found that these differences between the purines obtained after autolysis in different experiments were due to the fact that spleens from different animals were being studied, and he determined the presence of guanase in large amounts in the cow's spleen, although this ferment was entirely absent from the pig's spleen

⁸ Schulz *loc cit*

⁹ Oppenheimer's *Handbuch der Biochemie*, II, 2, p. 172, 1909

¹⁰ Capezuoli *Zeitschr f physiol Chem*, LV, pp. 10-14, 1909. Burov *Biochem Zeitschr*, XXV, p. 165, 1910

¹¹ Mandel and Levene *This Journal*, III, p. LVIII, 1907-08

¹² *Zeitschr f physiol Chem*, XLVIII, p. 300, 1906

¹³ Levene *Amer Journ of Physiol*, VI, p. 437, 1904, VII, p. 275, 1904-05

¹⁴ Jones *Zeitschr f physiol Chem*, XLII, p. 35, 1904

¹⁵ Jones *Ibid*, XLV, p. 84, 1905

Schumm¹⁶ autolyzed spleens from cases of myelogenous splenic leucemia and obtained free guanine, xanthine, and hypoxanthine

Schittenhelm,¹⁷ as a result of his investigations, states that the spleen of the cow contains a hydrolytic ferment which changes adenine of hypoxanthine, and guanine to xanthine, and an oxydase which forms xanthine from hypoxanthine but does not destroy uric acid. He analyzed one sterile dog spleen, which had been autolyzed in the ice chest and then kept in alcohol for one year, it yielded on hydrolysis xanthine and hypoxanthine but no adenine nor guanine. Wells and Corper¹⁸ have previously made note of the fact to be reported in this paper, that the dog spleen contains no uricolytic ferment, and observed that the human spleen contains no xanthine oxydase. Batelli and Stern,¹⁹ using their method of determining uricolysis by gaseous exchange, were also unable to demonstrate uricase in the dog spleen. Jones and Austrian²⁰ found that normal dog spleen contained guanase, adenase and xanthineoxydase and was to this extent similar to cow spleen. Burian and Schur²¹ obtained 0.16 gram of purine N from 100 grams (moist weight) of calf spleen, which was divided into 0.046 gram of free purine nitrogen and 0.101 gram of combined purine base nitrogen. Kossel²² found in the horse spleen 0.175 per cent purine nitrogen. Jones and Winternitz²³ observed that upon autolysis of the swine spleen there was a conversion of hypoxanthine into xanthine in the absence of air. Pohl²⁴ states that the normal spleen of starving dogs contains no allantoin, but that after autolysis allantoin appears. He does not, however, give his experiments with autolyzed spleen. This does not agree well with the absence of uricase noted by other authors.

¹⁶ Schumm *Hofmeister's Beiträge*, vii, p 175, 1905

¹⁷ Schittenhelm *Zeitschr f physiol Chem*, xlv, p 84, 1905

¹⁸ Wells and Corper *This Journal*, vi, p 321, 1909

¹⁹ Batelli and Stern *Biochem Zeitschr*, xix, p 219, 1909

²⁰ Jones and Austrian *Zeitschr f physiol Chem*, xlviii, p 110, 1906

²¹ Burian and Schur *Pflüger's Archiv*, lxxx, p 309, 1900

²² *Zeitschr f physiol Chem*, vi, p 422, 1882

²³ Jones and Winternitz *Ibid*, xlv, p 1, 1905

²⁴ Pohl *Arch f exp Path u Pharm*, xlviii, p 367, 1902

EXPERIMENTAL DATA

Analysis of Dog's Spleen

METHODS—The tissues were preserved for analysis in the ice chest in ten parts or more by weight of 95 per cent alcohol, samples for water content having been taken, and when ready for analysis the alcohol was filtered off to be mixed with the rest of the alcohol and ether extracts. The total ether extract was examined quantitatively for cholesterol by Ritter's method²⁵ and for lecithin by the method suggested by Koch and Woods²⁶. The combined residues after ether extraction were then pulverized and extracted in a shaking machine with N free water, containing alternately traces of alkali (sodium carbonate) and acid (acetic), and after bringing to faint acidity the combined watery extracts were concentrated to 1 liter and filtered hot, thus constituting the water-soluble fraction. This fraction was analyzed for purine content by the method of Kruger and Salomon,²⁷ and was also analyzed for a tannic acid precipitable fraction and a fraction not precipitable by tannic acid, and its phosphorus content was determined by the Neumann method as described by Koch and Woods. The remaining residue, after removal of ether and water extracts, was analyzed for its total nitrogen content by the Kjeldahl method, and for iron, phosphorus and sulphur by the method described by Koch and Mann.²⁸ Purine nitrogen was also determined on this residue after hydrolyzing, by means of 5 per cent sulphuric acid, using the Kruger and Salomon method. The Hausmann fractions were also determined on this tissue residue according to the modification described by Osborne and Harris.²⁹

NORMAL SPLEEN A *Results of Analyses*—Three small spleens weighing 19.25, 31.5 and 21.5 grams, with a moisture content of 76.82 per cent, 76.50 per cent and 76.64 per cent respectively, making a total dry weight of 16.87 grams, were taken. Total ether soluble material weighed 2.609 grams, or

²⁵ *Zeitschr f physiol Chem*, LVIV, p 461, 1903

²⁶ Koch and Woods. *This Journal*, I, p 203, 1906

²⁷ Hoppe-Seyler-Thierfelder. *Handbuch d physiol u pathol chem Analyse*, 8th edition, p 188, 1909

²⁸ Koch and Mann. *Archives of Neurol and Psychiatry*, IV, p, 20, 1903

²⁹ Osborne and Harris. *Journ of the Amer Chem Soc*, LV, pp 323-325,

15.47 per cent Absolute alcohol insoluble part of this amounted to 0.2783 gram, containing 0.00082 gram of phosphorus after Neumann oxidation. The cholesterol determination was lost in this analysis.

The lecithin phosphorus found in 1 gram dry weight tissue was 0.00269 gram, figured as lecithin = 0.0694 gram.

Non-lecithin phosphorus in this fraction = 0.0005 gram in 1 gram dry tissue.

Water Soluble Fraction

Nitrogen precipitable by tannic acid in 1 gram dry tissue = 0.00567 gram

Nitrogen not precipitable by tannic acid in 1 gram dry tissue = 0.00396

Water soluble N in 1 gram, total = 0.00972

Water soluble phosphorus in 1 gram dry tissue = 0.0052

Purines found in water soluble fraction, only a doubtful trace.

Tissue Residue (Insoluble) Fraction One gram of tissue (after extraction of soluble constituents) yielded 0.0035 gram of iron, 0.0070 gram of sulphur and about 0.0084 gram of phosphorus.

One gram of dry tissue residue (used 8.4 grams for analyses) yielded 0.00345 gram of purine nitrogen.

Total nitrogen determination (using 0.28 gram tissue residue) yielded 0.1447 gram of nitrogen in one gram of tissue residue.

NORMAL SPLEEN One large spleen with a moist weight of 134 grams, and a moisture content of 75.59 per cent, making a total dry weight of 32.71 grams was taken.

Total ether-soluble material weighed 3.8155 grams or 11.65 per cent.

Absolute alcohol insoluble part of this = 0.6361 gram, containing 0.0140 gram of phosphorus (after Neumann oxidation).

One gram dry tissue contained 0.015 cholesterol by the Ritter method (using one-half of the total ether extract for the analysis).

The lecithin fractions were lost in this analysis.

Water Soluble Fraction

Nitrogen precipitated by tannic acid in 1 gram dry tissue = 0.00412

Nitrogen not precipitated by tannic acid in 1 gram dry tissue = 0.00393

Water soluble N in 1 gram dry tissue, total = 0.00804

Water soluble phosphorus in 1 gram dry tissue = 0.00346

Purines found in water soluble fraction = a doubtful trace.

Tissue Residue (Insoluble) Fraction One gram of tissue residue (freed from soluble constituents) yielded 0.00455 gram of iron, 0.00714 gram of sulphur, and 0.00459 gram of phosphorus.

One gram of dry tissue residue (used duplicates of about 6 grams each) yielded 0.00292 gram of purine nitrogen.

Total nitrogen determination (using about 0.25 gram tissue residue in duplicates) yielded 0.1639 gram of nitrogen in one gram of tissue residue.

NORMAL SPLEEN C One large spleen with a moist weight of 82 grams and a moisture content of 76.94 per cent making a total dry weight of 18.91 grams.

Total ether soluble material weighed 2.859 grams or 15.11 per cent.

Absolute alcohol insoluble part of this = 0.2021 gram, containing 0.00339 gram of phosphorus (after Neumann oxidation)

Cholesterol determination was far too low, due to loss occasioned by the Ritter method, which will be discussed in a future paper

The lecithin phosphorus found in 1 gram dry tissue = 0.00241 gram figured as lecithin = 0.0622 gram

Non-lecithin phosphorus in this fraction = 0.00015 gram in 1 gram dry tissue

Water Soluble Fraction

Nitrogen precipitated by tannic acid in 1 gram dry tissue = 0.0012

Nitrogen not precipitated by tannic acid in 1 gram dry tissue = 0.00338

Total water soluble N in 1 gram dry tissue = 0.00458

Water soluble phosphorus in 1 gram dry tissue = 0.0027

Only doubtful trace of purines was found in the water soluble extractives

Tissue Residue (Insoluble) Fraction — One gram of tissue residue yielded 0.0122 gram of iron, 0.00745 gram of sulphur, and 0.00489 gram of phosphorus

One gram of dry tissue residue (used about a 6 gram sample) yielded 0.00428 gram of purine nitrogen

Total nitrogen determination (using about 0.26 gram, tissue residue in duplicates) yielded 0.1629 gram of nitrogen in 1 gram of tissue residue

Hausmann Fractions on tissue residue C — Duplicate analyses were obtained with the following result, from 1 gram of dry tissue residue

Amid N = 0.01349 and 0.01329 gram N — mean = 0.01339

Humus N = 0.00938 and 0.00751 gram N — mean = 0.00844

Diamino N = 0.03546 and 0.03244 gram N — mean = 0.03395

Monamino N = 0.1007 and 0.0969 gram N — mean = 0.09880

Total N = 0.15458

Purines and Purine Enzymes in Dog Spleen

I HYDROLYSIS OF DOG SPLEEN — 1091 grams of dog spleen (moist weight) were hydrolyzed by means of 5 per cent sulphuric acid and the purines isolated in the pure state and weighed, with the following results, figured on the basis of 1 gram moist weight of the original spleen tissue used

Guanine = 0.00109 gram (weighed as such)

Adenine = 0.00062 gram (weighed as pierate)

Hypoxanthine = 0.00015 gram (weighed as silver nitrate combination)

Xanthine = 0.00004 gram (weighed as such)

Total products = 0.00190 gram

No uric acid was found, although tested for

A purine nitrogen figure was obtained (using one-eightieth of the total material) and yielded in the figures of 1 gram moist weight of the original spleen tissue, 0.00126 gram of nitrogen

Nitrogen figure obtained from the total amount of isolated purines was 0 000899 gram per 1 gram moist splenic tissue

II AUTOLYSIS OF DOG SPLEEN IN THE ABSENCE OF AIR—552 grams of dog spleen (moist weight) were autolyzed for about a month, part of the time at 37° C and using toluene as antiseptic and to keep out air The autolysate was examined quantitatively for liberated purines and gave the following results, on the basis of 1 gram moist weight of original spleen tissue used.

No guanine, adenine or uric acid was found

The major portion of the purine product was

Xanthine = 0 00168 (weighed as such, and free from uric acid)

Hypoxanthine = 0 00002 gram (weighed as silver nitrate salt)

Total products = 0 00171 gram

Calculated purine nitrogen in these products = 0 00063 gram N, indicating that about half the total purine nitrogen was present in the form of free purines

III AUTOLYSIS OF DOG SPLEEN IN THE PRESENCE OF AIR—617 grams of dog spleen (moist weight) were autolyzed for about a month, part of which time a current of air was passed through the autolyzing mixture, and the temperature frequently brought to 37° C toluene being used as antiseptic The autolysate was examined quantitatively for purines, with the following results, on the basis of 1 gram moist weight of original spleen tissue used

Neither guanine or adenine were found

The major portion of the purines was

Uric acid = 0 001695 gram (weighed as such Repurified from concentrated H₂SO₄)

Xanthine = 0 000094 gram (weighed as such)

Hypoxanthine = 0 000004 gram (weighed as hypoxanthine silver nitrate)

Total products = 0 001793 gram

Calculated purine nitrogen in these products = 0 0006 gram or about half the total purine nitrogen

IV XANTHINE-OXIDASE OF DOG SPLEEN—Fifty grams of finely ground fresh dog spleen, mixed with three volumes of toluene water, was allowed to stand at room temperature over night, and strained through cheese cloth the following morning To this spleen extract thus obtained was added 0 1355 gram of xanthine in solution, and the mixture was placed in a bottle with sufficient toluene to prevent putrefaction, and connected with another bottle containing toluene and water, through which was drawn the air that then passed through the digestion mixture, and all the bottles were kept at about 40° C for twenty-four hours After autolysis 0 0969 gram of uric acid was recovered, which gave a positive murexide test, and upon repurification from H₂SO₄, pure uric acid was obtained, thus differentiating it from xanthine

V URICASE OF DOG SPLEEN (*Experiment 1*)—Sixty-three grams of ground up dog spleen, and 50 grams of ground up dog's liver as control (high uricolytic power of dog liver having been demonstrated) were each mixed with three volumes of saturated toluene water and strained through cheese cloth after standing over night at room temperature. To the spleen extract thus obtained was added a solution of 0.1735 gram of uric acid, and to the liver extract was added 0.1616 gram of uric acid. Both were then kept at about 40° C with a supply of air passing through them, for 48 hours and they were then analyzed for uric acid. From the spleen extract was recovered 0.1919 gram of uric acid, while the liver extract did not contain a trace of uric acid.

Experiment 2—Eighty grams of ground up dog spleen were allowed to stand at room temperature over night, and was then strained through cheese cloth. The extract was divided into two equal portions. To No. I was added 0.1655 gram uric acid in solution, and to No. II, after boiling fifteen minutes was added 0.1962 gram uric acid in solution. Both were then kept at about 40° C for twenty-four hours, during which time they received a supply of air bubbling through them and were then analyzed for uric acid content. From No. I was recovered 0.1687 gram uric acid (which when recrystallized from H_2SO_4 yielded 0.1638 gram) and from No. II which had been boiled, was recovered 0.1856 gram of uric acid.

SUMMARY

1 Average of analyses of three normal dogs' spleens resulted as follows

A moisture content of about 75 to 77 per cent

A content of ether-soluble materials between 11.6 and 15.5 per cent of the dry weight, which was made up of about 1.5 per cent cholesterol and between 6 and 7 per cent lecithin (leaving but 2 to 6.5 per cent neutral fats)

The total soluble nitrogen ranged between 0.45 per cent and 0.97 per cent of the dry weight, divided about equally between that precipitable and that not precipitable with tannic acid

A water-soluble phosphorus content of about 0.27 to 0.52 per cent

No purines were found in the water-soluble fraction, at least not in sufficient quantity from the amounts of tissue used to be recognized as such

The insoluble part of the tissue contained about 0.26 to 0.98 per cent of dry weight as iron, 0.53 to 0.60 per cent of dry weight as sulphur, and about 0.39 per cent of dry weight as phosphorus,

with a purine nitrogen content of 0.24 to 0.35 per cent of the dry weight

The total nitrogen content of the insoluble part was about 11.0 to 13.4 per cent of the dry weight, which was distributed as follows: Amid N, 8.60 per cent, Humus N, 5.76 per cent, Diamino N, 21.87 per cent, and Monamino N, 63.71 per cent

In contrast to normal liver, examined by Wells³⁰ by the same method, the insoluble residue of the spleen contained a slightly larger percentage of its dry weight as iron, about the same percentage of sulphur and a greater percentage of phosphorus. The Hausmann nitrogen fractions were also slightly different, more amid nitrogen, about the same or a little more humus nitrogen, less diamino nitrogen and about the same percentage of monamino nitrogen

2 The purines obtained from 1 kilo (moist weight) of dog spleen after hydrolysis were: Guanine, 1.09 gram, adenine, 0.62 gram, hypoxanthine, 0.15 gram, and xanthine 0.04 gram. Analyzed purine nitrogen content in one kilo moist weight = 1.62 grams

3 The purines obtained from the autolysate from 1 kilo (moist weight) dog spleen, after autolysis in the absence of air, was 1.69 grams of xanthine and 0.017 gram of hypoxanthine

4 The purines obtained from the autolysate from 1 kilo (moist weight) of dog spleen, upon autolysis in the presence of air, were 1.69 grams of uric acid, 0.09 gram of xanthine and 0.004 gram of hypoxanthine

5 Of the purine enzymes, evidence was obtained of the presence of xanthine oxidase, adenase and guanase, while uricase was lacking. The conversion of hypoxanthine to xanthine during comparative anaerobic conditions indicates the presence of an oxidizing enzyme with this particular function, whether it be the xanthine oxidase itself or a special hypoxanthine oxidase

³⁰ Wells. *This Journal*, v, pp 141-142 1903

ERRORS IN THE QUANTITATIVE DETERMINATION OF CHOLESTEROL BY RITTER'S METHOD THE INFLUENCE OF AUTOLYSIS UPON CHOLESTEROL ¹

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While carrying out analyses of normal and autolyzed spleens reported in previous papers³ the Ritter method⁴ for determining cholesterol quantitatively was found inadequate. The results were so variable for the amounts used that it was thought advisable to investigate the steps in the method in order to find the source of error, and also to find out whether the method actually could be used for the quantitative determination of cholesterol in tissues. At least one other investigator, Helen Baldwin,⁵ has had difficulty in the quantitative determination of cholesterol by this method.

The quantitative methods for cholesterol determination suggested up to 1908 (a discussion of these methods is given by Gilkin⁶) practically all depend either upon the saponification of the fat to be examined, the cholesterol being recovered as such, or upon the esterification of the cholesterol and the determination of the iodine figure or saponification number.

More recently Windaus⁷ has suggested a new method for the determination of cholesterol and cholesterol esters, precipitating

¹ This work has been aided by a grant from the Rockefeller Institute for Medical Research.

A portion of this work was done in the Physiological Laboratory of the University of Illinois.

³ Cf. preceding article on The Chemistry of the Dog's Spleen and reference 23, page 44.

⁴ E. Ritter *Zeitschr f. physiol. Chem.*, **xxiv**, p. 461, 1903.

⁵ Helen Baldwin *This Journal*, **iv**, p. 213-219 (218), 1908.

⁶ W. Gilkin *Biochem. Centralbl.*, **vii**, pp. 289-306, 357-77, 1908.

⁷ A. Windaus *Zeitschr f. physiol. Chem.*, **lxviii**, pp. 110-117, 1910.

them by means of digitonin Lapworth⁸ reports good results using this method

In order to more clearly understand where loss in Ritter's method might occur, the literature was searched for studies on the action of the various chemicals used in the method upon cholesterol in forming salts, or actual decomposition products and the properties of these compounds

Lindenmeyer⁹ prepared sodium cholesterolate, which he purified from chloroform by cooling the solution on ice, and found it to be insoluble in water, and that it was only slowly decomposed by water, weak alcohol hastening this decomposition Obermuller¹⁰ prepared potassium cholesterolate and found the properties similar to those of the sodium compound Darmstadter and Lifschutz¹¹ were able to obtain oxidation products of cholesterol by heating cholesterol with alcoholic potash with a reflux condenser for eight hours, the yield being 20 to 25 per cent Lifschutz¹² divided the products into three phases (1) Oxycholesterin ethers, (2) Oxycholesterins and (3) Dicarbonsaure (Chollansaure), the first two being soluble in all the ordinary solvents except water, and the last being soluble in water and alkalis and precipitating as white flocks on acidification Schulze and Winterstein¹³ noted a drop in the melting point of cholesterol exposed to the light apparently due to oxidation, as an atmosphere of CO₂ prevented this change

Lifschutz¹⁴ states that one hour's cooking with half-normal alcoholic potash does not alter cholesterol E Schulze¹⁵ recovered cholesterol from substances in which potash did not liberate it so that it could be extracted by ether, by heating with benzoic acid in sealed tubes and forming an ester insoluble in alcohol and ether

⁸ A Lapworth *Journ of Path and Bact*, xv, pp 254-61, 1911

⁹ Lindenmeyer *Erdmann's Praktische Chemie*, xc, pp 321-332, 1863

¹⁰ Obermuller *Zeitschr f physiol Chem*, xv, pp 37-48, 1891

¹¹ L Darmstadter and J Lifschutz *Ber d deutsch. chem Ges*, xxi, pp 1122-27, 1898

¹² J Lifschutz *Zeitschr f physiol Chem*, l, pp 436-40, 1906-07

¹³ E Schultz and E Winterstein *Ibid*, xliii, pp 316-19, 1905, xlviii, pp 546-48, 1906,

¹⁴ J Lifschutz *Ibid*, lviii, p 175, 1908

¹⁵ E Schulze *Zeitschr f anal Chemie*, xlii, p 173

Very little reliable work has been done on the changes occurring in cholesterol during autolysis, probably in part because of the fact that no reliable quantitative cholesterol method has been available, and partly because the change occurring, if such does occur, is naturally a slow one. Windaus,¹⁶ method for determining cholesterol and cholesterol esters, and Lifschutz's¹⁷ recent investigations upon the determination of the presence of oxidation products of cholesterol, should make research fruitful along these lines.

Moore¹⁸ failed to find any change in the cholesterol content of the liver in autolysis under toluene for forty-two days at 37°C (Cholesterol analyzing 0.038 per cent before and 0.0372 per cent after autolysis). He also found no significant difference between the cholesterol content of a normal area (0.64 per cent) and an infarcted area (0.58 per cent) in a human spleen, and strongly objects to the reasoning of Carbone,¹⁹ who believes that cholesterol originates from lecithin by decomposition, and of Waldvogel²⁰ who claims to have established the same by digesting lecithin with sterile liver juice, and who also found an increased cholesterol content in pathological livers as compared to normal (normal cholesterol content being 0.42 per cent, pathological—acute poisoning—being 24.46 per cent according to these analyses).

EXPERIMENTAL PART

METHODS Ritter puts 50 grams of fat into a porcelain dish, adds 100 cc of alcohol, brings it to a boil on the water bath, and then adds 8 grams of sodium dissolved in 160 cc of 99 per cent alcohol with constant stirring. (The sodium alcoholate is prepared according to the method described by Kossel and Krüger.²¹ These authors bring the absolute alcohol to a boil under a reflux condenser and carefully add the metallic sodium to it while boiling. They state that 10 cc of a 5 per cent sodium alcoholate

¹⁶ Windaus *loc cit*

¹⁷ J Lifschutz *Zeitschr f physiol Chem*, lxx, pp 140-48, 1907

¹⁸ F Craven Moore *Medical Chronicle*, xlii, pp 204-40, 1907-08

¹⁹ Tito Carbone *Arch ital de biol*, xvi, p 279, 1896

²⁰ Waldvogel and Mette *Münch med Woch*, lxx, p 402, 1906

²¹ A Kossel and M Krüger *Zeitschr f physiol Chem*, vi, p 321, 1891

solution thus prepared will saponify 5 grams of mutton tallow, and 15 cc will saponify 5 grams of butter fat)²² The alcohol is then evaporated off on the water bath and about one and one-half times as much salt as fat used is added, and enough water so that most of the contents of the evaporating dish goes into solution. This is then dried on the water bath with constant stirring, and then at 80°C, in a drying oven. It is pulverized, put into a sulphuric acid dessicator for a short time, then into an extraction thimble, and is extracted in a Soxhlet apparatus with ordinary ether for nine hours. The ether extract is then put into a separatory funnel and shaken out with water to remove glycerin. The ether extract is dried, dissolved in hot alcohol, precipitated by means of water, precipitate dried at 100-120°C and weighed.

Experiments on the Effect of Sodium Alcoholate upon the Quantitative Yield of Cholesterol by Rutter's Method

In order to test the loss occasioned by the steps in the method, the following experiments were carried out, using pure cholesterol instead of a complex fat mixture.

EXPERIMENT 1 The amount of cholesterol which can be recovered from salt mixture, being mixed in alcohol solution, dried, and extracted by means of absolute ether in a Soxhlet apparatus, and the ether extract shaken out by means of water.

(a) Used 0.1016 gram cholesterol and recovered 0.1026 gram

(b) Used 0.1020 gram cholesterol and recovered 0.1030 gram

EXPERIMENT 2 Amount of cholesterol which can be recovered from salt mixture after treating with sodium alcoholate, evaporating to dryness, dissolving residue in ether and shaken out by means of water.

(a) Used 0.1057 gram cholesterol mixed with 5 cc of 5 per cent sodium alcoholate and recovered 0.1048 gram cholesterol

(b) Used 0.1007 gram cholesterol mixed with 10 cc of 5 per cent sodium alcoholate and recovered 0.1016 gram cholesterol

(c) Used 0.1005 gram cholesterol mixed with 40 cc of sodium alcoholate and recovered 0.0950 gram cholesterol

(d) Duplicate of (c) Used 0.1003 gram cholesterol and recovered 0.1020 gram cholesterol

EXPERIMENT 3 Amount of cholesterol which can be recovered after solution in absolute alcohol, saponification by means of 10 cc of sodium

²² The sodium alcoholate employed in the following experiments was prepared according to this method, 5 per cent strength being used.

alcoholate, evaporated to dryness, mixing with salt (10 to 15 grams), and extraction by means of absolute ether in a Soxhlet for 9 hours, shaking out ether extract with water etc

(a) Used 0 1013 gram cholesterol and recovered 0 0390 gram

(b) Used 0 1012 gram cholesterol and recovered 0 0437 gram

EXPERIMENT 4 Similar to Experiment 3 but using only 5 cc of sodium alcoholate

(a) Used 0 1016 gram cholesterol and recovered 0 0347 gram

(b) Used 0 1042 gram cholesterol and recovered 0 0358 gram

EXPERIMENT 5 Similar to Experiment 3 but using 40 cc of sodium alcoholate

(a) Poured on salt in absolute alcohol solution, after saponifying

Used 0 1014 gram cholesterol and recovered 0 0415 gram

(b) Duplicate of (a) Used 0 1016 gram cholesterol and recovered 0 0390 gram

(c) Evaporated to dryness after saponification and emulsified by means of water, mixed with salt, dried, extracted with ordinary ether, etc

Used 0 1032 gram cholesterol and recovered none

(d) Duplicate of (c) Used 0 1046 gram cholesterol and recovered none

(e and f) Not evaporated to dryness after saponification, emulsified with saturated salt solution, dried, ground up, extracted with absolute ether, etc

Used 0 1023 gram cholesterol and recovered 0 0163 gram

Used 0 1002 gram cholesterol and recovered 0 0150 gram

EXPERIMENT 6 Amount of cholesterol that can be recovered after saponification by means of 40 cc of sodium alcoholate, evaporating as nearly dry as possible, dissolved in ether and in water and these extracts poured on salt and dried and then extracted in a Soxhlet by means of absolute ether, etc

(a) Used 0 1010 gram cholesterol and recovered 0 0770 gram

(b) Used 0 1003 gram cholesterol and recovered 0 0700 gram

As a result of the above experiments we can conclude that even an excess of 5 cc of 5 per cent sodium alcoholate added to cholesterol, will prevent its complete extraction from a dried salt mixture by means of ether

That the trouble lies in the use of an excess of sodium alcoholate is further shown by the following experiments

In order to simplify matters the following abbreviation is used for the various steps

A Cholesterol dissolved in absolute alcohol and heated on water bath for three days

B Saponifying with sodium alcoholate

- C Mixing with NaCl and drying
 D Extracting in a Soxhlet with absolute ether
 E Shaking out the ether extract with water in a separatory funnel
 F Allowing the ether to evaporate off at room temperature, and drying the residue at 100° C and weighing

TABLE OF RESULTS

Cholesterol

COMBINATIONS	USED	RECOVERED	LOSS
	<i>gram</i>	<i>gram</i>	<i>gram</i>
A F	0 1068	0 1075	0 00
E F	0 1001	0 1013	0 00
A C D E F	0 1001	0 0969	0 0032
B C D E F	0 1096	0 0955	0 0141
A B C D E F	0 1061	0 0675	0 0386

In the above experiments the exact amount of sodium alcoholate used was not noted, as they were carried out before the first set cited

Now if an excess of sodium alcoholate thus affected the yield of cholesterol by the Ritter method when pure cholesterol was used, what would be its effect upon the cholesterol yield from tissues? In this case we are unable to tell the exact amount of fats and esters present, and therefore the amount of sodium alcoholate necessary to saponify them. If we use too small an amount our result will be high, due to the unsaponified fats and esters remaining as such with the cholesterol, and if we use too much the cholesterol yield will be low. To test these points the following experiments were carried out in the ether and alcohol extract from a steer spleen

The steer spleen weighed about 850 grams (moist) and yielded an alcohol and ether extract weighing 29 13 grams, which was dissolved in a liter of absolute alcohol and divided in 50 cc samples (5 per cent of the total extract) for the following analyses for cholesterol

Cholesterol

EXPERIMENT 1 The extract after evaporation was dissolved in 10 cc of absolute alcohol and warmed, 10 cc of 5 per cent sodium alcoholate was added and the mixture warmed several hours, evaporated to dryness, redissolved in absolute alcohol, poured on 10 to 15 grams of salt, dried, extracted in a Soxhlet with absolute ether

Results (a) Yielded 0.0263 gram cholesterol

(b) Yielded 0.0195 gram cholesterol

EXPERIMENT 2 Identical with Experiment 1 except that the saponified mixture was poured directly on the salt (without evaporation)

Results (a) Yielded 0.0162 gram cholesterol

(b) Yielded 0.0380 gram cholesterol

EXPERIMENT 3 Identical with Experiment 2 except 30 cc. of absolute alcohol was used as a solvent before saponification

Results (a) Yielded 0.0215 gram cholesterol

(b) Yielded 0.0528 gram cholesterol

EXPERIMENT 4 Identical with Experiment 2, but used 40 cc. of sodium alcoholate and evaporated the saponified mixture as nearly to dryness as possible, redissolved and poured on salt

Results (a) Yielded 0.0072 gram cholesterol

(b) Yielded 0.0066 gram cholesterol

(c) and (d) were not evaporated after saponification before adding to the salt

(c) Yielded 0.0050 gram cholesterol

(d) Yielded 0.0100 gram cholesterol

EXPERIMENT 5 Identical with Experiment 1 except that only 5 cc. of sodium alcoholate was used

Results (a) Yielded 0.1773 gram cholesterol

(b) Yielded 0.1600 gram cholesterol

EXPERIMENT 6 The object of this experiment was to compare an old preparation of sodium alcoholate (five months old) and of dark brown color with the freshly prepared compound. It is practically a duplicate of Experiment 5 but using 5 cc. of old sodium alcoholate

Results (a) Yielded 0.1624 gram cholesterol

(b) Yielded 0.1840 gram cholesterol

EXPERIMENT 7 Resembled Experiment 5 except in that the mixture was not evaporated to dryness after saponification but poured directly on the salt after standing 24 hours

Results (a) Yielded 0.1306 gram cholesterol

(b) Yielded 0.1312 gram cholesterol

EXPERIMENT 8 Identical with Experiment 1 but used 3 cc. 5 per cent sodium alcoholate (The resulting product was only slightly oily in appearance)

Results (a) Yielded 0.2180 gram cholesterol

(b) Yielded 0.2155 gram cholesterol

EXPERIMENT 9 Identical with Experiment 8, but used only 1 cc. of sodium alcoholate (The product was not crystalline but oily)

Results (a) Yielded 0.3543 gram cholesterol

(b) Yielded 0.2956 gram cholesterol

As a result of the above experiments we can conclude that the best yield of cholesterol is obtained from the alcohol and ether

extract of the spleen when about 5 cc of 5 per cent sodium alcoholate is used to saponify 1.5 gram of the ether-alcohol extract, we must, however, expect an error in the quantitative results on this amount of extract of from 10 per cent to 25 per cent

Cholesterol in Autolysis

Remembering the possibility for analytical error by the Ritter method as shown above, the following experiments can carry no great weight, but will merely be cited to show that there is no marked change in the cholesterol content of the spleen (dog) during autolysis

EXPERIMENT 1 Sixty-five grams of ground dog spleen were mixed with 0.338 gram of cholesterol suspension (made by dissolving the cholesterol in a minimum amount of absolute alcohol and pouring it into 0.9 per cent sodium chloride solution), toluene was used as preservative and the mixture allowed to autolyze for ninety-two hours at room temperature

Recovered 0.591 gram of cholesterol or 0.253 gram above the amount added which must have come from the spleen (Extraction, etc., was carried on here as in the case of the steer spleen analyses)

EXPERIMENT 2 Sixty-five grams of ground up dog spleen was mixed with 0.314 gram cholesterol (suspended in 0.9 per cent NaCl-toluene water) The mixture autolyzed at room temperature for fifty-three hours

Recovered 0.5566 gram cholesterol or 0.242 gram of cholesterol over the amount added

As a few successful analyses for cholesterol were obtained while carrying out the autolysis experiments reported in previous papers²³ they may be put into tabulated form for comparison with the two above mentioned experiments For the sake of convenience the figures will be given in the form of the amount of cholesterol found in one gram dry weight of spleen (on a basis of 23 per cent of solids in fresh spleen) In Experiments 1 and 2 above only the cholesterol content of the splenic tissue is given (that obtained by deducting the cholesterol added)

²³ Work to be published in the *Journal of Experimental Medicine* upon correlation of chemical and histological changes

SPLEEN	TIME AUTOLYZED	CHOLESTEROL PER 1 GRAM DRY WEIGHT SPLEEN
		gram
Normal (Spleen B Ref 3)	0 00	0 0150
Experiment 2 (above)	53 hours	0 0162
Experiment 1 (above)	92 hours	0 0169
*Six days autolysis (spleen H, Ref 3)	6 days	0 0216
*Two days <i>in vivo</i> autolysis (Spleen I, Ref 2)	48 hours <i>in vivo</i>	0 0217

*These two spleens were analysed at the same time the normal spleens above being analysed at an earlier period

The last two cholesterol figures though differing from the first three by about 20 per cent are still within the error limit of the method

In conclusion we can say then that within the limit of error of the Ritter method for cholesterol, this constituent of the tissues does not markedly change in amount during autolysis

GENERAL SUMMARY

1 A source of error was found in the quantitative estimation of cholesterol by the Ritter method, in the fact that the presence of an excess of sodium alcoholate over that necessary for the saponification of the fats and esters, prevents a complete extraction of the cholesterol from the salt mixture by means of ether

2 This error may vary from 5 per cent to 20 per cent in the case of a normal tissue when there is an excess of from 1 cc to 3 cc of a 5 per cent sodium alcoholate solution used in the saponification of 1.5 grams of the alcohol-ether extract

3 The Ritter method for the quantitative determination of cholesterol in tissues should be used only with certain restrictions and precautions in mind

4 No marked change was found in the amount of cholesterol present in the dog spleen after *in vitro* and *in vivo* autolysis of short duration

5 The steer spleen contains about 0.4 per cent of its moist weight as cholesterol

THE HAEMAGGLUTINATING AND PRECIPITATING PROPERTIES OF THE BEAN (*Phaseolus*).

By EDWARD C SCHNEIDER

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(Received for publication December 4, 1911)

The extracts of a number of kinds of seeds are capable of producing *in vitro* an agglutination and sedimentation of the red blood corpuscles of various animals. This peculiar property is largely confined to species of the Leguminosae and to a few Solanaceae, although an occasional member of other families may possess it. The property was first noted among certain toxic seeds, the several species of *Ricinus*, *Abrus pectoratus*, and *Croton tiglium*.² In recent years the list has been enlarged by a careful search for haemagglutinin bearing seeds. Landsteiner and Raubitschek³ found this property in extracts of beans, *Phaseolus*, peas, *Pisum*, vetches, *Vicia*, and lentils, *Ervum*, and v. Eisler and v. Portheim⁴ report its presence in five species of *Datura*. Mendel⁵ added the following: sweet pea, *Lathyrus odoratus*, lentil, *Lens esculenta*, yellow locust, *Robinia pseudacacia*, five species of *Vicia*, *Wistaria Chinensis*, *Caragana arborescens*, senna, *Cassia Marilandica*, and sweet rocket, *Hesperis matronalis*. He also found among beans that the haemagglutinins are absent in the Lima

¹ Most of the work here reported was done in the Sheffield Laboratory of Physiological Chemistry of Yale University. The writer wishes to express his hearty thanks to Professor Lafayette B. Mendel for the suggestion of the problem and for his kindly interest.

² For the early literature on these see Jacoby. *Biochemische Centralblatt*, i, p. 289, 1903.

³ Landsteiner and Raubitschek. *Centralblatt für Bakteriologie*, 1. Abteilung, 44, pp. 660-67, 1907.

⁴ v. Eisler and v. Portheim. *Zeitschrift für Immunitätsforschung und experimentelle Therapie*, i, p. 151, 1908.

⁵ Mendel. *Archivio di fisiologia*, vii, pp. 168-177, 1909.

bean Wienhaus⁶ reports that this property occurs in the soy bean, *Glycine* or *Soja hispida*, and Assmann⁷ found it in the seeds of *Canavalia ensiformis*, *Datura stramonium*, and three species of *Lathyrus*

The agglutinative property is not necessarily coincident with the toxic activity of seeds. It varies greatly in the seeds known to contain haemagglutinin and does not manifest itself equally well with the blood of different kinds of animals. Among laboratory animals Mendel⁸ reports the blood of the rabbit to be most susceptible, and those of the pig and the sheep the most refractory. The extract of a number of the seeds noted above reacts well with rabbit's blood but gives negative results with all other bloods tested. The reaction is strongest with suspensions of serum-free corpuscles. Landsteiner⁹ found the normal blood serum of many kinds of blood capable of checking the process but that agglutination occurred readily when washed corpuscles were used.

Several workers have suggested methods for obtaining purified preparations of the agglutinins from the crude extracts. Landsteiner and Raubitschek¹⁰ found that (1) the addition of a little acid produced a precipitate which contained only a trace of the agglutinin, the chief portion remaining in the filtrate. (2) When alcohol was added an agglutinative precipitate was obtained. It was also observed that when this precipitate was redissolved there was no loss of power. (3) The agglutinin was also salted out with the proteins on saturation with ammonium sulphate.

From the extract of beans Wienhaus¹¹ separated a mixture of proteins to which he has applied the name of Phasin. Ten grams of bean meal were extracted with 500 grams of 0.9 per cent sodium chloride solution for twenty-four hours and then filtered. To the filtrate an equal volume of alcohol was added. A voluminous precipitate of albumin and globulin was secured in which the agglutinin is held quantitatively. On drying this precipitate in a

⁶ Wienhaus *Biochemische Zeitschrift*, xviii, pp 228-60, 1909

⁷ Assmann *Pflüger's Archiv*, cxxxvii, pp 489-510, 1911

⁸ Mendel *Loc cit*

⁹ See Raubitschek *Hamagglutinine pflanzlicher Provenienz und ihre Antikörper*, Kraus and Levadite's *Handbuch der Technik und Methodik der Immunitätsforschung*, p 625, 1911

¹⁰ Landsteiner and Raubitschek *Loc cit*

¹¹ Wienhaus *Loc cit*

vacuum he secured a white powder which yielded to physiological salt solution all of the agglutinin and some inactive proteins. He suggests that he hopes later to free the "Phasin" from proteins by digestion.

Landsteiner¹² employed the characteristic of erythrocytes that causes them to give up to the suspension fluid, when gently heated, the agglutinins with which they are combined. To this end he agglutinated, in an ice chest, sensitive serum-free corpuscles with purified bean extract for several hours. The corpuscles were then washed with cold isotonic salt solution in a centrifuge until no trace of agglutinin was found in the washing solution. The agglutinated corpuscles were next suspended in a small amount of salt solution and stirred for an hour at 45° C. With precautions to avoid cooling they were then centrifugalized. By this means he obtained a clear but often red colored solution containing the agglutinin. This he found he could further purify by dialysis or with ammonium sulphate.

Thus far the nature of these vegetable haemagglutinins has not been satisfactorily determined. Landsteiner and Raubitschek conjecture it to be a protein by analogy with the very pure ricin isolated by Osborne, Mendel, and Harris¹³. The latter investigators separated the proteins of the castor bean, *Ricinus zanzibarensis*, by dialysis and fractional precipitation with neutral salts and found the physiological properties, toxic and haemagglutinative, to be associated with the coagulable albumin. The agglutinative action was absent in the globulin and proteose fractions, and very active in the albumin fractions.

SEPARATION OF THE PROTEIN CONSTITUENTS OF THE BEAN

In view of the experience of Osborne, Mendel, and Harris an attempt has been made to separate the haemagglutinin of the Scarlet Runner bean, *Phaseolus multiflorus*, Willd. A preliminary examination of a number of varieties of beans was made for the purpose of determining which is richest in haemagglutinins.

¹² See Raubitschek in Kraus and Levadite's *Handbuch der Technik und Methodik der Immunitätsforschung*, p 625, 1911.

¹³ Osborne, Mendel and Harris *American Journal of Physiology*, xiv, pp 259-86, 1905.

Among these were the dwarf wax-podded varieties Burpee's Kidney, Wardell's Kidney Wax, Red Kidney, Dwarf Champion, and Early Six Weeks, and the climbing wax-podded variety Golden Champion of *P vulgaris*, L., also the Scarlet Runner, *P multiflorus*, Willd. The extracts prepared from equal weights of bean meal were almost equally active. The Scarlet Runner seed is much larger than the seeds of the other varieties which favored the removal of the seed coat.

The Scarlet Runner beans were first passed through a very coarse grinder. Much of the seed coat was thus broken away from the substance of the cotyledons and was blown out with an air blast. Afterward these cracked beans were ground to a coarse meal and treated with benzine to remove the oil. Following this the coarse meal was ground to a powder and 1 kilo of it was extracted with 5 liters of a 2 per cent sodium chloride solution that had been previously heated to 60° C. After frequent stirrings for two hours it was placed in a cold room over night and then filtered perfectly clear. The extract was dialyzed in running water for thirty-six hours. The precipitate I which separated was filtered from the solution B and dried. Unfortunately precipitate I was dried so slowly that more than two-thirds of it was changed into an insoluble protean.

Solution B was further dialyzed three days and yielded a heavy precipitate II which, when dried, was more than 85 per cent soluble. The solution while dialyzing tended to become acid in reaction and required frequent neutralization. It was protected against decomposition with toluene.

Solution B₁, which remained after filtering off precipitate II, was again dialyzed four more days and yielded a small amount of precipitate III. Precipitates I and II were the globulin phaseolin, and III was probably the other globulin, phaseolin, separated by Osborne¹⁴ from the kidney bean.

The proteins remaining in solution B₂ (obtained from B₁ on filtering off precipitate III) were salted out by saturating with ammonium sulphate. This procedure yielded precipitate IV and solution B₃. Solution B₃ was then dialyzed in running water until free from salts when it was found it did not contain a trace of the haemagglutinin.

Precipitate IV was dissolved in a small volume of water and the clear solution C was then saturated with magnesium sulphate and weakly acidulated with acetic acid. The small amount of precipitate V, which will be called albumin, was then filtered from solution C₁.

The albumin precipitate, V, was redissolved and precipitated with magnesium sulphate and then dissolved in a very small volume of water. From this solution the salt was removed by dialysis. The solution was next evaporated at a low temperature and yielded 0.3 gram of albumin.

¹⁴ Osborne *Journal of the American Chemical Society*, 16, p. 635 and p. 707, 1894.

Solution C1 was dialyzed for several weeks until free from salt, then was evaporated in low dishes at 48° C. Almost a gram of proteoses was secured from this solution.

THE ACTION OF THE BEAN PROTEIN PREPARATIONS ON BLOOD

Tests were always made with defibrinated rabbit's blood diluted (1:5) with 0.9 per cent sodium chloride solution. One cubic centimeter of this blood mixture was placed in a small and very narrow test tube, and 2 cc. of the protein preparation, dissolved in the salt solution, were added. The time of the visible beginning of agglutination and the condition at the end of two, four, and twelve hours were noted.

Preliminary tests with the phaseolin and phaselin (preparations I, II, and III) revealed the presence of haemagglutinin. Preparation II was most active but none of the globulin preparations exhibited the property in a striking degree. Believing that these proteins adsorbed the haemagglutinin, an attempt was made to purify precipitate II. About half of preparation II was dissolved in 0.9 per cent sodium chloride solution. One-half of this solution was dialyzed until it yielded its phaseolin, preparation IIa, and the other half of the solution was saturated with magnesium sulphate. The resulting precipitate was redissolved in water and on dialysis yielded preparation IIb. Preparations IIa and IIb were less active than preparation II. This weakening in activity by purification indicates that the haemagglutinin in these preparations is held there by adsorption.

The albumin, purified from precipitate V, was also active but less so than the globulins. The degree of activity of the albumin and globulins is given in Table I.

The proteose preparation was found to be rich in haemagglutinin. It produced strong agglutination when present in blood dilutions of one part to 100,000 and more. Wienhaus¹⁵ found his crude product "Phasin" completely agglutinated rabbit's blood in the ratio of 1:7000 in fifteen hours, and with cat's blood, which reacted still better, in dilutions of 1:11,000 in eighteen hours and 1:60,000 in twenty-three hours. Assmann¹⁶ also working with a

¹⁵ Wienhaus *Biochemische Zeitschrift* xxvii, p. 232-33, 1909.

¹⁶ Assmann *Pflüger's Archiv*, cxviii, pp. 489-510, 1911.

TABLE I

Agglutination Trials with Protein Preparations

PREPARATION USED	MILLIGRAMS ADDED TO 1 CC OF BLOOD MIXTURE	FIRST INDICATION OF AGGLUTINATION	REMARKS
No I Phaseolin	5 000	2 minutes	Complete in 2 hours
	1 000	25 minutes	Partial in 12 hours
	3 000	At once	Complete in 2 hours
	0 600	2 minutes	Complete in 4 hours
No II Phaseolin	0 300	5 minutes	Complete in 12 hours
	0 200	15 minutes	Complete in 18 hours
	0 150		Trace
	0 100		Negative
No IIa Phaseolin	0 600	4 minutes	Complete in 4 hours
	0 300	10 minutes	Complete in 12 hours
	0 200	(?)	Partial in 12 hours
	0 150		Negative
No IIb Phaseolin	1 500	2 minutes	Complete in 12 hours
	0 750	(?)	Complete in 18 hours
	0 300		Negative
No III Phaseolin	3 000	4 minutes	Complete in 4 hours
	0 600	(?)	Trace
No V Albumin	3 200	4 minutes	Complete in 12 hours
	0 610		Negative
	0 300	At once	Complete in 2 hours
	0 060	At once	Complete in 2 hours
Proteose	0 020	2 minutes	Complete in 4 hours
	0 015	15 minutes	Complete in 12 hours
	0 0075	19 minutes	Complete in 18 hours
	0 0037	(?)	Partial in 18 hours

"Phasin" preparation obtained agglutination of diluted rabbits blood in 1 35,000. The rapidity of action and the dilutions of the proteose preparation that are effective are given in the latter part of Table I. This preparation is very soluble and gives the distinctive proteose tests. One milligram of the proteose dissolved in 1 cc of salt solution added to 5 cc of undiluted defibrinated rabbit's blood produced almost instantaneous agglutination, and a solid clot-like mass of corpuscles settled out leaving a clear serum in less than half an hour. Table II gives further testimony as to the power of the haemagglutinin associated with the proteose.

TABLE II

MILLIGRAMS OF PROTEOSE ADDED		VISIBLE AGGLUTINATION	COMPLETE AGGLUTINATION AND SEDIMENTATION
5 cc of 1.5 blood	1.0 in 1 cc 0.9 per cent NaCl	At once	20 minutes
	0.5 in 1 cc 0.9 per cent NaCl	At once	45 minutes
	0.4 in 1 cc 0.9 per cent NaCl	At once	1 hour
	0.3 in 1 cc 0.9 per cent NaCl	At once	2 hours
	0.2 in 1 cc 0.9 per cent NaCl	2 minutes	6 hours
	0.1 in 1 cc 0.9 per cent NaCl	4 minutes	Incomplete in 6 hours*

* Further observation was impossible because it then stood over night at room temperature. All but the last gave a firm clot like mass in the time recorded.

It seemed probable, in view of the observation that the haemagglutinin was largely confined to the proteose preparation, that all proteoses might cause agglutination. Hence tests were made with Witte's peptone upon the diluted rabbit's blood corpuscles but these were entirely negative.

DOES AUTOLYSIS ACCOUNT FOR THE HAEMAGGLUTININ?

The presence of the haemagglutinin in the proteose preparation also suggested that it might be a product of the hydrolysis occurring in the solution during the period of extraction and later. To settle this point a fresh extract was prepared as rapidly as possible and immediately tested for its agglutinating power. A portion of the extract was also immediately heated for five minutes at 82° C—a temperature the haemagglutinin withstands for thirty minutes without injury¹⁷—the coagulated proteins were filtered off and the filtrate was then tested for the relative amount of haemagglutinin. There was slightly less in this than in the original extract as is shown in Table III. This is very likely due to a slight adsorption by the coagulated proteins. The remaining portion of the original extract, after the addition of toluene, was set aside in a cool room for thirty days. Its agglutinating power was again determined on the eighth and thirtieth days. There was not a decided change in agglutinating power as will be observed

¹⁷ Wienhaus *Loc cit*

TABLE III

EXTRACT DILUTED WITH 0.9 PER CENT NaCl*	FRESH EXTRACT	AFTER HEATING FIVE MINUTES AT 82° C	EXTRACT EIGHT DAYS OLD	EXTRACT THIRTY DAYS OLD
Undiluted	Complete	Complete	Complete	Complete
1 100	Complete	Complete	Complete	Complete
1 200	Complete	Complete	Complete	Complete
1 300	Complete	Complete	Complete	Complete
1 400	Complete	Partial	Partial	Complete
1 500	Partial	Slight	Partial	Complete
1 600	Partial	Negative	Slight	Partial

* Two cubic centimeters of extract and 1 cc of 1:5 blood used in each test. Agglutination recorded at end of twelve hours.

in Table III. It was also found that active agglutinins may be secured by extracting the bean meal at 80° C. From these observations it would seem that autolysis does not account for the haemagglutinin in the proteose preparation.

Digestion trials. Osborne, Mendel, and Harris¹⁸ showed that the toxicity and agglutinating power of their pure preparation of ricin could be impaired or destroyed by pancreatic digestion prolonged two or three months. Wienhaus,¹⁹ on the other hand, in digestive trials with pepsin, trypsin, and papain made on his "Phasin" for periods ranging from three to seven days failed to show any destructive action.

The haemagglutinative proteose preparation was subjected to various digestive trials with trypsin, erepsin, and mixtures of these two, in water and in sodium carbonate solutions for a period of twenty-eight days with practically negative results. The digestive mixtures were tested with fresh blood fibrin and Witte's peptone several times during the period and found to be active. Wienhaus calls attention to the fact that his "Phasin" acts as a protein and he expresses the opinion that it is a protein or enzyme-like substance. Since Wienhaus' digestion trials were so very short and the effective trials of Osborne, Mendel, and Harris were so prolonged the failure of the proteose preparation to respond to digestive agents in the time allowed still leaves the question of the

¹⁸ Osborne, Mendel and Harris. *American Journal of Physiology*, **xiv**, p 284, 1905.

¹⁹ Wienhaus. *Biochemische Zeitschrift*, **xviii**, p 256, 1909.

digestibility of these haemagglutinins open. A more prolonged series of carefully controlled digestive trials is planned for the near future.

IS THE HAEMAGGLUTININ A FOOD STORED FOR THE USE OF THE GROWING SEEDLING?

If the haemagglutinin of the seed is a proteose it should readily be utilized by the growing seedling in the early growth after germination. It is also probable that preliminary to the translocation of the protein from the cotyledons to the growing tissues of the seedling further haemagglutinin may be formed from the proteins by the action of the enzymes evolved during germination. It certainly is surprising to find the haemagglutinins in the proteose portion of the seed, inasmuch as proteoses and peptones are not commonly normal constituents among the reserve proteins of seeds. They are of course present to some extent during germination. It may be noted here that Osborne²⁰ found a small amount of proteoses when he studied the proteins of the kidney bean. He did not determine whether the proteoses were a normal constituent of the seed or a product of autolysis during extraction. Landsteiner and Raubitschek²¹ showed the agglutinin to be absent from green beans. A future study must determine when the haemagglutinin enters the seed and an attempt be made to learn its source, whether it is formed in the seed or brought to it to be stored.

To determine if the haemagglutinin is utilized by the seedling and whether it is increased in amount during germination a study was made of seedlings and cotyledons at frequent intervals, from the beginning of germination until the depleted cotyledons fell from the seedling. Two series of observations were made, one with plants grown in darkness and the other with sturdy plants grown in the light. For the determination of haemagglutinin content the seedlings were hastily washed and the cotyledons separated from the seedling close to the stem, and then cotyledons and seedlings dried separately. When dry each was ground to a powder and known weights extracted with constant proportions of a 0.9

²⁰ Osborne *Journal of the American Chemical Society*, **xvi**, pp 758-64 1894

²¹ Landsteiner and Raubitschek *Loc cit*

per cent sodium chloride solution Three kinds of beans were used, the Scarlet Runner, Wardell's Kidney, and the Early Six Weeks The cotyledons of the last two are lifted above the soil by the growing stem of the seedling while those of the Scarlet Runner remain underground The underground habit of the Scarlet Runner made it difficult to secure from the late stages cotyledons that had not undergone decomposition to some extent The data obtained from the three kinds of beans were wholly concordant throughout each series

Repeated tests with colorless seedlings and with the green leaves and stems of those grown in the light failed to show the slightest trace of agglutinative power Hence the haemagglutinin as such is not carried into the seedling or, at least, not in sufficient amounts to be detected Roots, stems, and leaves were also examined separately from plants of many sizes, all being negative The agglutinin is not a normal constituent of the organs of the vegetative plant

As a type of the results obtained, those with the cotyledons of the Wardell's Kidney bean are given in Table IV, p 57 During the early days of growth the agglutinative action for equal weights of cotyledon is only slightly lowered, which indicates that the haemagglutinins are withdrawn gradually along with other stored foods Later there is a more rapid disappearance of the agglutinin Extracts prepared from cotyledons that fell from the seedlings of Wardell's Kidney bean and Early Six Weeks bean, grown in darkness, gave no agglutinative response From seedlings of all three varieties when grown in light, and the Scarlet Runners grown in darkness, it was impossible to get depleted cotyledons wholly free from the haemagglutinins With each, however, there was a very marked quantitative reduction in this property It follows, therefore, that the haemagglutinin of the bean is utilized or destroyed, along with other stored foods, by the developing seedling

THE PRECIPITATING REACTION OF BEAN EXTRACTS

When the clear extract of any of the several beans examined in this study was added to rabbit's blood serum a flocculent precipitate always appeared The reaction usually occurred slowly

TABLE IV

*Wardell's Kidney Bean During Germination and Early Growth
Grown in Light*

WEIGHT OF TWENTY COTYLEDONS	LENGTH OF SEEDLING	GREATEST DILUTION AT WHICH AGGLOUTINATION WAS OBTAINED
grams	centimeters	
6 000	0	1.550
0 918	9 0	1.350
0 606	13 2	1.300
0 300	17 8	1 100
0 262	*	Undiluted

Grown in Darkness

1 000	9 4	1 400
0 508	13 2	1 200
0 245	18 3	1 150
0 215	35 5	Negative*

* Cotyledons that had fallen from seedlings

For some minutes, and often more than an hour, after the addition of the extract to the blood serum the mixture remained clear. It then gradually became cloudy and opaque, finally the white flocculent precipitate appeared. The entire reaction may be completed within a few minutes when strong extracts are used but will require five or more hours with dilute extracts.

This precipitating reaction is not constantly associated with the agglutinative property of seed extracts. It was found to be absent in extracts from such agglutinin containing seeds as the *Wistaria Chinensis*, the hairy vetch, *Vicia villosa*, and the pea, *Pisum sativum*. From the sweet pea, *Lathyrus odoratus*, an extract was obtained that gave a slight clouding of the serum but it failed to produce a precipitate.

A fresh extract prepared from Scarlet Runner bean meal was heated repeatedly at various temperatures for five-minute intervals, and after each period of heating the coagulated proteins were filtered off and the filtrate then tested for the agglutinating and precipitating properties. Both properties continued practically undiminished up to a temperature of 80° C. At 83° C the precipitating power was destroyed in ten minutes. Table V

TABLE V

CONDITION OF EXTRACT	PRECIPITATE IN SERUM	AGGLUTINATION OF CORPUSCLES
Fresh	Heavy in 50 minutes	Strong
After heating at 80° for five minutes	moderate in 4 5 hours	Strong
After heating at 83° for five minutes	Trace in 7 hours	Strong
After heating at 85° for five minutes	Negative	Strong
After heating at 87° for five minutes	Negative	Strong
After heating at 91° for five minutes	Negative	Strong
After heating at 94° for five minutes	Negative	Negative

shows a trace present after five minutes at 83° The agglutinative power was weakened above this temperature, but withstood five minute exposures to 91°, and was wholly destroyed at 92° Table V contains the data obtained from one series of heat tests

The protein preparations separated for the study of the agglutinins have also been tested for the precipitin reaction The globulin preparations II, IIa, and IIb were rich in it while I contained a trace The albumin (V) gave a negative test, and 3 mgm of the proteose preparation in 2 cc of serum failed to give the reaction

It was also found that after serum had been added repeatedly to extract until no more precipitate formed that the mixture retained its agglutinating power practically unaltered

These several differences warrant the conclusion that the precipitating and agglutinating properties of the extracts of beans are due to different constituents of the seed Or we may better express it that rabbit's blood contains a precipitin for certain of the bean's proteins

Wienhaus^{2*} made certain observations which are of interest in this connection He found his "Phasin" did not react with serum taken from hen's blood On adding the preparation to a clear fluid collected from the joint of a diseased knee a heavy precipitate was obtained After immunizing rabbits to the phasin it was impossible to obtain a precipitate in the blood serum on the addition of phasin He points out that this is contrary to the experience of Jacoby and others when they immunized animals to ricin, abrin, and crotin, inasmuch as these substances gave a precipitate when added to the immune sera It would seem from Wienhaus' work that the agglutinating and precipitating properties are both lost for the blood on immunizing the animal

* Wienhaus *Loc cit*

The precipitating property does not occur in extracts of bean plants. It disappears from the cotyledons, as does the agglutinin, with germination and the growth of the seedling.

SUMMARY

1 The proteose prepared from the Scarlet Runner bean was found to be a very active haemagglutinating agent. Other bean proteins contained some haemagglutinin but this was shown to be adsorbed by them.

2 The haemagglutinin is not a product of autolysis.

3 The haemagglutinin gradually disappears from the cotyledons, simultaneously with the stored food, as the seedling develops.

4 The agglutinative property does not occur in the extracts of the roots, stems, or leaves of the bean plant.

5 The addition of the clear extract of beans to rabbit's blood serum produces a flocculent precipitate. This reaction is not coincident with the agglutinating property of all haemagglutinin containing seeds and appears to be chiefly associated with the phaseolin in the bean.

Since this paper was written there has been brought to my attention an abstract, in the *Zentralblatt für Biochemie*, xii, p 391, 1911, of a recent paper by v. Eisler and v. Portheim. They regard the haemagglutinin as a protein and prove it to be a reserve substance that disappears from the embryo during germination.

ON THE RECOVERY OF ALCOHOL FROM ANIMAL TISSUES

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In the course of some experiments on the absorption of alcohol, I have found it advantageous to introduce certain modifications in the current distillation method

The procedure which is herewith described differs only in a few points from those already in use, but these points are rather important They involve (1) the digestion of the tissue with phosphoric acid to liberate the alcohol and to facilitate its distillation, (2) the automatic filtration of the distillate to remove the volatile solid products which would interfere with the specific gravity determination, and (3) a more delicate ring modification of Anstie's test, to determine the completion of the distillation

The method is as follows

The organ or tissue (15 to 300 grams) is placed in a distilling flask of 1000 cc capacity and 50 per cent phosphoric acid (5 to 25 cc) is added, together with 300 cc of water

The flask is then connected with a condenser In the distal end of the condensing tube a plug of dry absorbent cotton is packed rather firmly The flask is heated over a direct flame or sand bath until the distillate no longer gives a bluish or light green color with Anstie's test made by contact A distillate of 200 cc usually suffices with small quantities of alcohol

The distillate, which is free from insoluble matter, is stirred by gentle rotation of the flask and carefully measured and its specific gravity determined with a pyknometer at 25° C , in the usual manner The alcohol percentage is calculated from the alcohol specific gravity tables of the United States Pharmacopoeia

The modifications were adopted for the following reasons

1 *The addition of phosphoric acid* This has two advantages it liquefies the tissues and thus hastens the distillation, and it makes the recovery of the alcohol more complete, increasing the yield by about 13 per cent The quantitative data will be discussed later

2 *The automatic filtration of the distillate through cotton* Distillates from animal tissues, particularly from the alimentary tract, contain some white flaky material consisting of fatty acids, indol, skatol, etc They are especially abundant if the tissue is acidified These falsify the readings of the pyknometer, and, therefore, had to be eliminated

Various modes of chemical treatment were tried to effect their removal, but with no practical success Sodium hydroxide prevented the volatilization of the fatty acids but the residue foamed so much that it was impossible to distil it Other alkalies such as calcium hydroxide and sodium carbonate had the same disadvantage In neither acid nor alkaline media was the volatilization of indol and skatol prevented Redistillation was ineffective

The object was finally accomplished by simple filtration of the distillate through filter paper or cotton This was combined with the distillation in the manner described above in order to avoid loss of alcohol by repeated handling of the distillates and to make the process as practical as possible In this way the distillates always appeared free from any insoluble matter and the pyknometer weighings were not affected

3 *The contact modification of Anstie's method* A practical qualitative method of detecting alcohol was needed, to insure the completeness of the distillation All the more common methods were tried, but the bichromate-sulphuric acid test¹ (also known as the Anstie test), modified so as to make it more delicate, was found to be the most useful If solutions containing very small quantities of alcohol are used, the green color obtained becomes too diffuse and can not be recognized To avoid this, the "contact-test" was performed as follows The solution containing the alcohol (about 1 cc) is placed in a test-tube, then the bichromate-sulphuric acid solution² (about 0.5 cc) is introduced by means of a pipette beneath

¹ Merck *Reagentien Verzeichniss*, Darmstadt, 1903

² Five-tenths of a gram of potassium bichromate dissolved in 75 grams of concentrated sulphuric acid

the alcohol layer taking care not to mix the solutions. At the point of contact a blue or light green ring will develop depending upon the concentration of the alcohol solution. After standing for a short time the ring becomes more intense but gradually fades away owing to diffusion and the establishment of equilibrium between the two liquids.

The other tests were performed in the usual manner. In all cases blank tests on distilled water were simultaneously performed. The results obtained are shown in Table I. Positive reaction is designated by +, negative by -.

TABLE I

TEST	BLANK	STRENGTH OF ALCOHOL USED			
		1:2000	1:5000	1:10000	1:100000
Bichromate-sulphuric acid (contact)	- (yellow)	+	+	+	+
Iodoform	-	+	+	+	-
Ethyl benzoate (Berthelot)	-	+	+	-	-
Ammonium molybdate	-	+	-	-	-

It can readily be seen that the bichromate-sulphuric acid test was the most sensitive. The color ring usually appeared quite promptly (within five minutes). With high dilutions of alcohol (1:10,000) a light green ring appeared in about ten minutes. Next in order of sensitiveness was the iodoform test. In high dilutions, however, it was difficult to differentiate the odor of iodine from iodoform and the result of a search for crystals was often unsuccessful. Least sensitive and reliable of all were the ethyl benzoate and molybdate tests. In high dilutions, it was impossible to differentiate the odor of benzoyl chloride from that of ethyl benzoate, while the molybdate gave an almost indistinguishable faint blue tint with the lowest dilution of alcohol.

Variable and uncertain results are to be expected in tests which require the olfactory sense. On the other hand, if properly carried out, an objective test, such as the bichromate-sulphuric acid test, is apt to give more constant and certain results under otherwise varying conditions.

Quantitative control tests The procedure here described, was tested out in the following manner Blood and intestines of cats and dogs were used The blood was intimately mixed with the alcohol The viscera, deprived of their circulation, were ligated at both ends and injected with different quantities of alcohols of known strengths The total quantity of alcohol varied between 0.6 and 5.1 grams The material was allowed to remain different lengths of time before the recovery of alcohol was begun The results are presented in Table II

TABLE II

EXPERIMENT	ABSOLUTE ALCOHOL USED	ABSOLUTE ALCOHOL RECOVERED	ALCOHOL RECOVERED	TISSUE OR ORGAN USED	REMARKS DISTILLED WITH
	grams	grams	per cent		
1	1.0245	1.0270	100.24	Half of whole intestine	Phosphoric acid
3	5.1100	4.8816	95.53	Blood 100 cc and half of intestine	Phosphoric acid
4	0.6108	0.6300	103.14	Blood 100 cc	Phosphoric acid
5	5.0900	4.9400	97.05	Blood 150 cc	Phosphoric acid
6	0.6108	0.6200	101.50	Intestine 15 cm	Phosphoric acid
10	1.4139	1.4080	99.58	Intestine 15 cm	Phosphoric acid
11	1.2568	1.2719	101.20	Intestine 15 cm	Phosphoric acid
12	1.2568	1.2640	100.57	Intestine 15 cm	Phosphoric acid
Average			99.85		
7	2.0360	1.9950	97.99	Whole intestine	Water alone
9	1.0180	1.0099	99.20	Intestine 15 cm	Water alone
Average			98.59		
8	1.0180	0.9986	98.10	Intestine 15 cm	Sodium hydroxide
Grand average			98.84		

An inspection of the table shows that distillation with phosphoric acid gave the highest results. The amount of alcohol recovered above that when water alone (Experiments 7 and 9) was used was about 1.3 per cent, and above that when sodium hydroxide (Experiment 8) was used about 1.8 per cent. Inasmuch as the individual results under varying conditions were quite comparable with the average (99.85 per cent), it would seem justifiable to conclude that the procedure is suitable for quantitative purposes. The best results were obtained when the quantity of alcohol did not exceed 2 grams.

The results obtained agree favorably with those reported recently by Bacon³ with the aid of the refractometer. The average of his six experiments was about 97.4 per cent with quantities of alcohol ranging from 0.95 gram to 8.0 grams in variable strengths. There were no animal tissues involved in the residues from which the distillates were obtained.

Hamill⁴ has reported a quantitative method for the determination of alcohol in perfusion fluids and tissues. The alcohol is recovered by distillation and estimated volumetrically in the distillate by sulphuric and chromic acids. Good results are claimed to have been obtained with quantities varying from 0.5 to 1 part *per mille*. Nicloux's⁵ method, also based on the principle of oxidation of alcohol by sulphuric acid and potassium bichromate, is said to be accurate for small quantities, the limit of error being about 5 percent, in more experienced hands somewhat less.

CONCLUSIONS

The modifications in the method of alcohol estimation in animal tissues, which are described in this paper, give results which are accurate within 1 per cent. The bichromate-sulphuric acid test reveals the presence of alcohol in dilutions of 1/10,000.

I wish to thank Professor Sollmann for his suggestions and criticisms in this work.

³ Bacon Circular No. 74, Bureau of Chemistry, U. S. Department of Agriculture, July 14, 1911.

⁴ Hamill *Journ. of Physiol.*, **xxix**, p. 476, 1910.

⁵ Abderhalden *Handbuch der biochem. Arbeitsmethoden*, **ii**, p. 7, 1909.

RESEARCHES ON PURINES

ON 2-OXYPURINE AND 2-OXY-8-METHYLPURINE

FOURTH PAPER ¹

By CARLO JOHNS

(From the Sheffield Laboratory of Yale University)

(Received for publication, December 8, 1911)

Hypoxanthine or 6-oxypurine (II) was the first of the monoxypurines to be described. This was isolated from ox-spleen by Scherer as early as 1850 ². This was almost a half century before Emil Fischer ³ synthesized hypoxanthine and showed that it is 6-oxypurine. 8-Oxypurine (III) has been prepared by Fischer and Ach ⁴. The third isomer, 2-oxypurine (I), was prepared by Tafel and Ach ⁵ from guanine. These workers reasoned from analogy that the compound which they obtained must be 2-oxypurine but they did not offer any direct proof of its structure. It may be for this reason or through an oversight that Fischer in the introduction to his book, *Untersuchungen in der Puringruppe*, page 49 (1907) states that 2-oxypurine is still unknown.

The writer has synthesized 2-oxypurine from 2-oxy-5,6-diaminopyrimidine and finds that it agrees in all respects with the description given by Tafel and Ach of their compound.

When 2-oxy-5,6-diaminopyrimidine ⁶ (IV) was heated with formic acid a monoformyl derivative was obtained. This yielded a potassium salt which, when heated, gave off water and changed to the

¹ *Amer Chem Journ*, xl, p 58, 1909, *Ibid*, xlv, p 79, 1911, *This Journal*, xv, p 161, 1911

² *Ann d Chem* (Liebig), lxxiii p 328, 1850

³ *Ber d deutsch chem Gesellsch*, xxx, p 2228, 1897

⁴ *Ibid*, xxx, p 2213, 1897

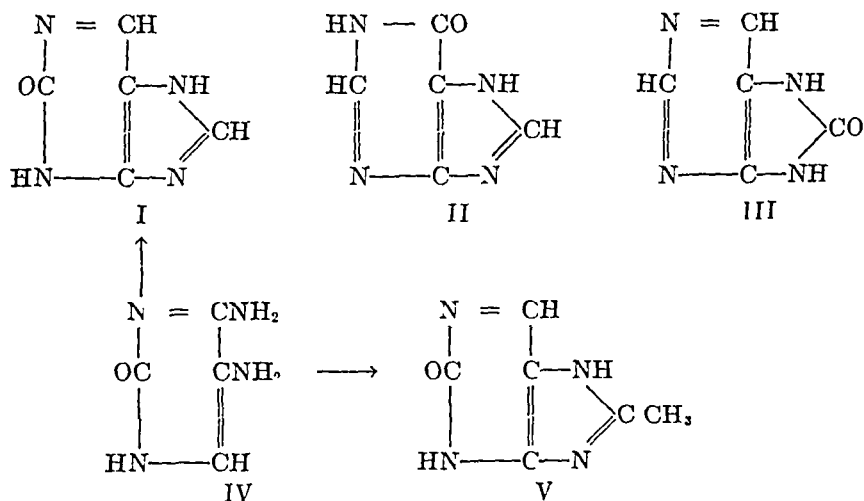
⁵ *Ibid*, xxxiv, p 1170, 1901

⁶ *Johns Amer Chem Journ* xlv, p 82, 1911

potassium salt of 2-oxypurine. As good yields were obtained, reasonable quantities of 2-oxypurine can be made in this manner. 2-Oxy-purine is characterized by the fact that it crystallizes with one molecule of water so firmly bound that it does not escape at 110°C . On heating at 130°C the crystals become anhydrous. Neither hypoxanthine nor 8-oxypurine crystallize with water. Of the salts of 2-oxypurine, the picrate, nitrate, and hydrochloride are easily prepared.

When 2-oxy-5,6-diaminopyrimidine is boiled with acetic anhydride it forms chiefly a monoacetyl compound together with some of the diacetyl compound. When the potassium salt of the monoacetyl compound is heated it yields the potassium salt of 2-oxy-8-methylpurine (V). This purine forms a picrate and a nitrate, both of which have rather definite decomposition points. These salts may therefore be used to identify this purine.

Work on the preparation of 2-oxy-1-methylpurine is almost completed and this compound will be described in a later paper.



EXPERIMENTAL PART

Formyl-2-oxy-5,6-diaminopyrimidine, $\text{C}_5\text{H}_6\text{O}_2\text{N}_4$

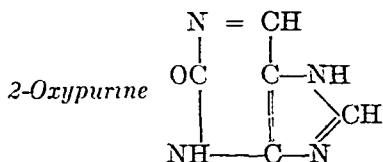
Four grams of 2-oxy-5,6-diaminopyrimidine⁷ were dissolved in 10 cc of 85 per cent formic acid. The solution was heated on

⁷ Johns *Loc cit*

the steam bath for an hour after which it was evaporated to dryness. The residue was treated with a little alcohol and evaporated again to remove the last traces of formic acid. It was then dissolved in dilute ammonia, a trace of insoluble material was filtered off and the filtrate was evaporated to dryness. A yield corresponding to 90 per cent of the calculated was obtained. The portion used for analysis was recrystallized from water and was obtained as a powder composed of aggregates of very minute crystals. These were easily soluble in hot and sparingly soluble in cold water, and almost insoluble in alcohol.

N

Calculated for	Found
$C_5H_4O N_4$	
36.36	36.33



When formyl-2-oxo-5,6-diaminopyrimidine was heated at 160°–170° C it blackened considerably and, although it was partly changed to 2-oxypurine, the reaction was unsatisfactory. A good yield of the purine could be obtained by heating the potassium salt of the formyl compound. This salt was made by dissolving the formyl compound in a small volume of water containing a little more than one molecular proportion of potassium hydroxide. Alcohol was added gradually to this solution to precipitate the salt which separated as a white powder. Five grams of this potassium salt were heated at 150°–160° C for an hour. Water was liberated, leaving a light brown crust. This was dissolved in water and the solution was decolorized with blood coal whereupon it was acidified with acetic acid. On standing over night, the solution gave a precipitate of small globules which in turn were found to be aggregates of very minute prisms. These were recrystallized from water. The yield was 70 per cent of the calculated. The crystals contained one molecule of water which they did not lose at 110° C. When heated at 130° C they became anhydrous. Analyses of samples dried at 110° C gave the following results.

- I 3 7600 grams of substance lost 0 4400 gram at 150° C
 II 2 6800 grams of substance lost 0 3100 gram at 150° C
 III 0 7792 gram of substance lost 0 0907 gram at 150° C

	Calculated for $C_5H_4ON_4 \cdot H_2O$	I	Found I.	III
H ₂ O	11 68	11 70	11 57	11 64
N	36 36	36 60		

0 2144 gram of anhydrous substance gave 0 0593 gram of H₂O and 0 3475 gram of CO₂.

	Calculated for $C_5H_4ON_4$	Found
C	44 11	44 20
H	2 94	3 07
N	41 17	41 28

The properties of 2-oxypurine agreed in all respects with the description of the purine which Tafel and Ach⁸ prepared from guanine

Salts

The Hydrochloride $C_5H_4ON_4 \cdot 2HCl$ One-half gram of anhydrous 2-oxypurine was dissolved in 10 cc of hot 20 per cent hydrochloric acid. On cooling rapidly the hydrochloride separated as slender prisms, but when the solution was cooled slowly rectangular plates were obtained. The precipitate weighed 0 3 gram.

0 1203 gram of substance gave 0 1641 gram of AgCl

	Calculated for $C_5H_4ON_4 \cdot 2HCl$	Found
Cl	33 97	33 73

The Nitric Acid Salt $C_5H_4ON_4 \cdot 2HNO_3$ One-half gram of the anhydrous 2-oxypurine was dissolved in 10 cc of warm 20 per cent nitric acid. Clusters of slender prisms separated rapidly on cooling. The yield was 0 5 gram.

	Calculated for $C_5H_4ON_4 \cdot 2HNO_3$	Found I	II
N	32 06	32 10	32 06

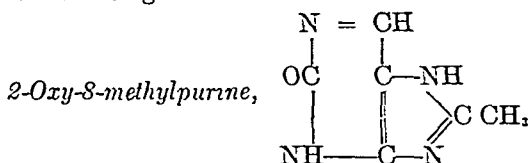
The Picrate $C_5H_4ON_4 \cdot C_6H_2(NO_2)_3OH$ This was made by adding a cold saturated solution of picric acid to a hot solution of the purine. On cooling, hexagonal and lenticular shaped prisms were obtained. These were easily soluble in hot and difficultly

⁸ Tafel and Ach *Loc cit*

soluble in cold water They turned brown when heated above 210° C and effervesced at 245° C

N	Calculated for	Found
	$C_6H_4ON_4$ $C_6H_3(NO_2)_3OH$	
	26 85	26 82

Acetyl-2-oxy-5,6-diaminopyrimidine $C_6H_5O_2N_4$ Six grams of 2-oxy-5,6-diaminopyrimidine were dissolved in 60 cc of acetic anhydride by heating at 140° C in an oil bath This solution was then evaporated to dryness on the steambath A little dilute ammonia was added to neutralize the last traces of acetic acid After evaporating to dryness the residue was washed with a little water The yield was greater than the calculated for monoacetyl-2-oxy-5, 6-diaminopyrimidine, a mixture of the mono- and diacetyl compounds having formed This mixture was moderately soluble in hot water from which it crystallized in slender prisms with square ends The calculated per cent of nitrogen for a monoacetyl compound is 33 33, for a diacetyl 26 66 The mixture contained 31 67 per cent of nitrogen



Three and one-half grams of acetyl-2-oxy-5,6-diaminopyrimidine were dissolved in 8 cc of water containing 2 5 grams of potassium hydroxide About 200 cc of absolute alcohol were added Finally ether was added until the precipitation was complete The potassium salt deposited as a thick oil which solidified on standing The salt thus obtained was heated in an oil bath at 240° C It melted partially and foaming ensued as water was liberated The heating was discontinued when steam ceased to escape The reaction product was a brittle crust This was dissolved in cold water and the solution was acidified with acetic acid and evaporated to dryness The potassium acetate was removed by washing with a little cold water The yield of the crude purine was 90 per cent of the calculated The yield varied widely in several experiments, the variation being probably due to the proportion of diacetyldiamino pyrimidine present The purine was purified by

dissolving in dilute ammonia and clarifying with blood coal. The filtrate was boiled to remove most of the ammonia whereupon it was acidified with acetic acid. The 2-oxy-8-methylpurine separated slowly from the solution in the form of small slender prisms with tapering ends. These were soluble in about 40 parts of boiling water and slightly soluble in cold water and 95 per cent alcohol. They turned brown at 285° C but did not decompose completely below 310° C.

0.1780 gram of substance gave 0.0671 gram of H_2O and 0.3123 gram of CO_2 .

	Calculated for $C_6H_6ON_4$	I	Found II	III
H	4.00	4.18		
C	48.00	47.87		
N	37.33	37.39	37.32	37.32

SALTS

The Nitric Acid Salt $C_6H_6ON_4 \cdot HNO_3$. One-half gram of 2-oxy-8-methylpurine was dissolved in 3 cc of 30 per cent nitric acid by warming gently. On cooling, the salt separated rapidly in minute lenticular crystals. When the solution was cooled slowly the crystals were more compact and had truncated ends. When heated rapidly in a capillary tube the salt began to turn dark at about 170° C and decomposed suddenly at 205° C with enough force to throw the substance out of the tube.

	Calculated for $C_6H_6ON_4 \cdot HNO_3$	Found
N	32.86	32.94

The Picrate. A cold saturated solution of picric acid was added to a hot solution of the purine. Sheaf-like clusters of slender prisms were deposited as the solution cooled. These were moderately soluble in hot water. They began to turn dark at about 210° C and decomposed with violent effervescence at 250° C.

	Calculated for $C_6H_6ON_4 \cdot C_6H_3(NO_2)_3OH$	Found
N	25.85	26.02

RESEARCHES ON PURINES ON 2-OXY-1-METHYLPURINE

FIFTH PAPER ¹

By CARL O. JOHNS

(From the Sheffield Laboratory of Yale University)

(Received for publication, December 14, 1911)

Five of the six isomers of 2-oxymonomethylpurine have been described. The first of these was obtained by Emil Fischer who made 2-oxy-7-methylpurine² (X) from 2-iodo-7-methylpurine. The same purine was also prepared by Tafel and Weinschenk.³ The latter workers also prepared 2-oxy-3-methylpurine⁴ (VIII). In all of the above cases the starting material was a purine. The remaining isomers have been synthesized from pyrimidines.

2-Oxy-6-methylpurine⁵ (IX) was prepared from 2-oxy-4-methyl-5,6-diaminopyrimidine. 2-Oxy-9-methylpurine⁶ (XII) was prepared from 2-oxy-6-methylamino-5-aminopyrimidine, while 2-oxy-8-methylpurine⁷ (XI) was made from 2-oxy-5,6-diaminopyrimidine.

The sixth isomer of this series, 2-oxy-1-methylpurine (VII) has now been synthesized from 2-oxy-3-methyl-5,6-diaminopyrimidine (IV). The reactions involved in this synthesis are as follows. The potassium salt of nitrocytosine, 2-oxy-5-nitro-6-aminopyrimidine⁸ (II) was methylated by the means of methyl iodide. The yield of a monomethyl derivative was 70 per cent of the calculated quantity. Three different monomethyl deriva-

¹ This *Journal*, vi, p 67, 1912

² *Ber d deutsch chem Gesellsch*, xxvi, p 2854, 1898

³ *Ibid*, xxviii, p 3376, 1900

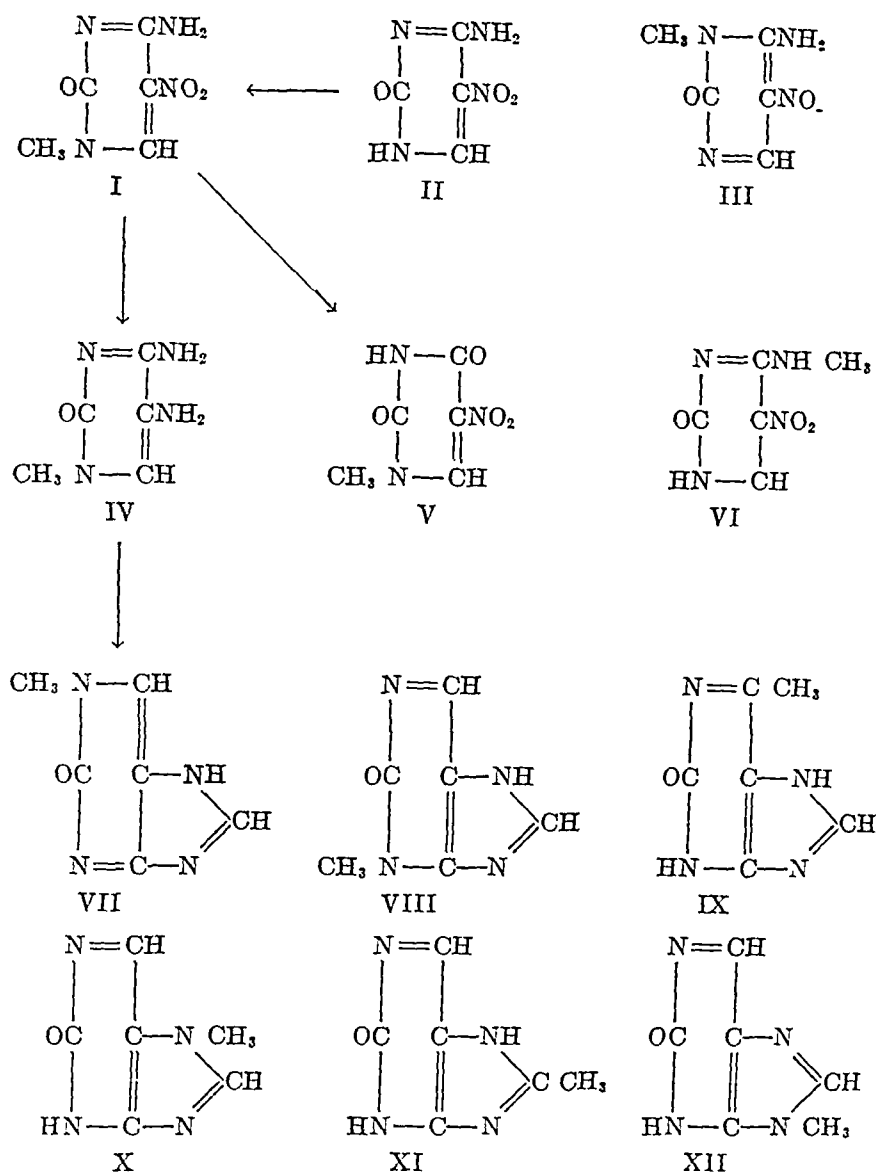
⁴ *Ibid*, p 3372

⁵ *Johns Amer Chem Journ*, xli, p 65, 1909

⁶ *Johns This Journal*, ix, p 161, 1911

⁷ *Johns This Journal*, vi, p 67, 1912

⁸ *Wheeler and Johnson Amer Chem Journ*, xxvi, p 591, 1905, *Johns Ibid*, xlv, p 81, 1911



tives are possible in this reaction, namely, 1-methylnitrocytosine (III), 3-methylnitrocytosine (I), and 6-methylnitrocytosine (VI). To determine which one of these was formed the reaction product was heated with sulphuric acid in a sealed tube. This treatment removed an amino group, giving a methyluracil melting at 255° C and containing one molecule of water of crystallization.

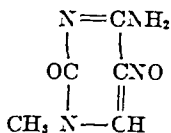
Hence formula VI was excluded. The two isomers of methyl-nitrouracil have been investigated by Behrend and Thurm.⁹ 1-Methylnitrouracil crystallizes without water and melts at 263° C while 3-methylnitrouracil (V) crystallizes with one molecule of water and melts at 255° C. Our compound was identical with the latter and hence our methylated product was 2-oxy-3-methyl-5-nitro-6-aminopyrimidine (I), or 3-methylnitrocytosine. When this compound was reduced with freshly precipitated ferrous hydroxide it gave a good yield of 2-oxy-3-methyl-5,6-diaminopyrimidine (IV), which, in turn, reacted with formic acid to give a formyl derivative whose potassium salt, when heated, lost water and formed the potassium salt of 2-oxy-1-methylpurine (VII).

2-Oxy-1-methylpurine crystallizes beautifully from water in flat prisms and these contain 2 molecules of water of crystallization. They effloresce in the air and become anhydrous over sulphuric acid. An aqueous solution of the purine gives difficultly soluble precipitates with platonic chloride and picric acid. The picrate decomposes at 214° C.

Work on the preparation of other purines from 2-oxy-3-methyl-5,6-diaminopyrimidine is in progress.

EXPERIMENTAL PART

2-Oxy-3-methyl-5-nitro-6-aminopyrimidine



This is the chief product where nitrocytosine¹⁰ is methylated as follows. Five grams of nitrocytosine, 2-oxy-5-nitro-6-aminopyrimidine, were dissolved in 50 cc of water containing 2 grams of potassium hydroxide. Five grams of methyl iodide were added and the mixture was heated in a sealed tube at 100° C for one hour. On cooling, the methylated product separated in slender prisms. The yield was 64 to 74 per cent of the calculated. The

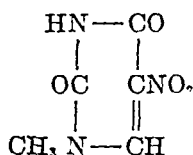
⁹ *Ann d Chem* (Liebig), cccviii p 163 1902

¹⁰ *Loc cit*,

compound was moderately soluble in hot and difficultly soluble in cold water, and difficultly soluble in hot alcohol or hot glacial acetic acid. It crystallized from water in beautiful, slender prisms which in some cases were about 1 to 2 mm thick and 3 cm long. These began to turn brown at 260° C and melted with decomposition at 274° C.

N	Calculated for	Found
	$C_8H_6O_2N_4$	
	32.93	32.72

2,6-Dioxy-3-methyl-5-nitropyrimidine



The position of the methyl group in the pyrimidine obtained by methylating nitrocytosine was ascertained as follows. One and five hundredths grams of the methylated product were heated with 18 cc of 25 per cent sulphuric acid in a sealed tube at 140 to 150° C for one and one-half hours. On cooling, 0.9 gram of crystals deposited from the acid solution. This substance was recrystallized from water and obtained in the form of slender prisms. These prisms melted at 255° C and contained one molecule of water and in all other respects agreed with the properties of 3-methylnitrouacil as described by Lehman¹¹ and by Behrend and Thurm¹². In this experiment the ammonia produced by heating the methyl derivative in the sealed tube was determined by making the acid solution alkaline and distilling the ammonia into $\frac{N}{10}$ acid, 62 cc being required.

N	Calculated for the loss of NH_3 in $C_8H_6O_2N_4$	Found
	8.23	8.27

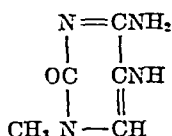
Analyses of the crystals obtained from the sealed tube gave the following results:

0.7303 gram of substance lost 0.0710 gram of H_2O at 120 to 130° C

¹¹ *Ann d Chem* (Liebig), 253, p 77, 1899

¹² *Loc cit*

H O	Calculated for $C_5H_5O_4N_4 \cdot H_2O$ 9 53	Found 9 72
N	Calculated for $C_5H_5O_4N_4$ 24 56	Found 24 83

2-Oxy-3-methyl-5,6-diaminopyrimidine

Ten grams of 2-oxy-3-methyl-5-nitro-6-aminopyrimidine were dissolved in a mixture of 200 cc of concentrated ammonia and 100 cc of water. To this solution was added a warm, almost saturated, aqueous solution of 120 grams of crystallized ferrous sulphate. Reduction took place rapidly with the liberation of heat. The sulphate was precipitated by the addition of 140 grams of crystallized barium hydroxide dissolved in hot water. After shaking thoroughly the excess of barium hydroxide was removed by the means of carbon dioxide. The reaction was allowed to proceed over night whereupon the precipitate was filtered off and washed with hot water. The filtrate was concentrated to about 30 cc. The diamino compound crystallized in small, stout, anhydrous prisms. These were easily soluble in hot and moderately soluble in cold water and almost insoluble in alcohol. They began to turn dark at about 220° C and decomposed slowly when heated above that temperature. The yield of the substance isolated was 82 per cent of the calculated, exclusive of a small quantity that remained in the mother liquor after further concentration.

N	Calculated for $C_5H_5ON_4$ 40 00	Found 39 77
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Formyl-2-oxy-3-methyl-5,6-diaminopyrimidine, $C_6H_5O_2N_4$

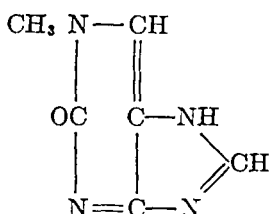
Eight grams of 2-oxy-3-methyl-5,6-diaminopyrimidine were dissolved in 20 cc of 85 per cent formic acid and the solution was evaporated to dryness on the steam-bath. The resulting residue was taken up in water, the solution was filtered to remove a little

insoluble substance, the filtrate was made slightly alkaline with ammonia and evaporated to dryness. The yield of formyl compound was almost quantitative. The compound was very soluble in hot and easily soluble in cold water. From a concentrated aqueous solution, it crystallized in masses of colorless, slender, distorted prisms.

N

Calculated for	Found
$C_5H_5O_2N_4$	
33.33	33.30

2-Oxy-1-methylpurine



The potassium salt of formyl-1-methyl-2-oxy-5,6-diaminopyrimidine was made by dissolving 4.5 grams of the formyl compound in 8 cc of water containing 3 grams of potassium hydroxide. Two hundred cubic centimeters of absolute alcohol were added but the salt did not precipitate. Finally ether was added, gradually, until the solution became turbid. On stirring, the salt began to crystallize. More ether was then added until crystallization was complete. Five grams of salt, dried at 80° C, were obtained. This salt was heated in an oil-bath at 160° C until water ceased to escape. A brittle crust remained. This was dissolved in water, the solution was neutralized with acetic acid and clarified with blood coal. On concentrating to about 15 cc the purine began to crystallize from the hot solution. The presence of potassium acetate appeared to render the purine easily soluble. It crystallized slowly in small, flat prisms. These contained two molecules of water and effloresced in the air. When dried over sulphuric acid for two days they became anhydrous. The anhydrous substance dissolved in about eight parts of boiling water. It was slightly soluble in hot alcohol and easily soluble in hot glacial acetic acid. From the latter it crystallized in small stout prisms. The aqueous solution gives precipitates with silver nitrate.

and platonic chloride The anhydrous purine decomposed slowly without melting when heated above 280°C

The portion used for analysis was recrystallized from water and the crystals were dried for two hours on filter paper

0.6950 gram lost 0.1350 gram of H_2O at 130 to 140°C

0.7359 gram lost 0.1421 gram of H_2O at 130 to 140°C

	Calculated for $\text{C}_6\text{H}_6\text{ON}_4$	Found	
		I	II
H	19.34	19.42	19.29
N	30.10	30.18	

0.2178 gram of anhydrous substance gave 0.0812 gram of H_2O and 0.3822 gram of CO_2

	Calculated for $\text{C}_6\text{H}_6\text{ON}_4$	Found
C	48.00	47.75
H	4.00	4.14
N	37.33	37.23

The Picrate, $\text{C}_6\text{H}_6\text{ON}_4 \cdot \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH}$

A cold saturated solution of picric acid was added to a hot solution of the purine. On cooling, clusters of small, slender prisms deposited. These were moderately soluble in hot and difficultly soluble in cold water. They melted with decomposition at 214°C

	Calculated for $\text{C}_{12}\text{H}_8\text{O}_5\text{N}_7$	Found
N	25.85	25.88

CONCERNING THE USE OF PHOSPHOTUNGSTIC ACID AS A CLARIFYING AGENT IN URINE ANALYSIS

By CLARENCE E MAY

(From the Department of Chemistry, Indiana University)

(Received for publication, December 15, 1911)

Until recently, the problem of clarifying a given urine containing a small amount of protein, so as to more accurately estimate the non-protein constituents, has been a more or less unsatisfactory thing. Usually it was necessary to resort to the older method that depended on heating the acidified urine to boiling. In the presence of a small but appreciable amount of protein this method was usually unsatisfactory, especially if the chemist was following the protein elimination with a glucose determination.

Various investigators have used phosphotungstic acid in the defecation of blood. E. Waymouth Reid¹ found it convenient to precipitate blood proteins by means of an hydrochloric acid solution of phosphotungstic acid. Vosburgh and Richards² used the Reid method very successfully in determining the glucose content of dog's blood after the injection of adrenalin chloride. Macleod³ has verified two methods, namely, the Reid method using phosphotungstic acid and the Schenck method using mercuric chloride as the protein precipitating reagents. Macleod found the Reid method to give better results with blood. The various chemists have used the original Reid method without any modifications, that is, the proteins were separated by the phosphotungstic acid, and the acid filtrates, on making alkaline with sodium hydroxide, were ready for the sugar determinations.

It seemed desirable to apply some defecation method to urines especially those containing much uric acid and creatinine as well

¹ Reid, *Journ of Physiol*, xx, p 316, 1896

² Vosburgh and Richards *Amer Journ of Physiol*, ix, p 38, 1903

³ Macleod *This Journal*, v, p 443, 1903-9

as small amounts of proteins. By the application of phosphotungstic acid it was hoped by the writer to be able to precipitate all the constituents of urine that interfered with the determination of the reducing sugars by Fehling's titration method. The work was carried out using phosphotungstic acid in hydrochloric acid solution as the precipitating reagent but there was delay in getting the results published. Meanwhile, a paper by Oppler⁴ has appeared in which the use of phosphotungstic acid as a defecating agent in urine as well as blood is pointed out. There were several differences in the two applications of the same precipitant and the present author felt inclined to publish his method and the results obtained.

In the Oppler method, the precipitation was brought about by the direct addition of solid phosphotungstic acid followed by heating to boiling. The excess of acid was gotten rid of by means of lead acetate in excess. The latter was removed by hydrogen sulphide, the excess of which was boiled out and the sulphur filtered. The filtrate, obtained many hours after the process started, was used for the sugar determination. In the author's method a given amount (50 cc) of urine was acidified with a few drops of concentrated hydrochloric acid and placed in a 150 cc graduated flask. At room temperature, the urine was treated with 50 cc of a 2 per cent of phosphotungstic acid solution. The mixture was diluted to the mark and filtered. Of the filtrate, 100 cc were measured into a 200 cc graduated flask, made neutral or barely alkaline with barium hydroxide solution, diluted to the mark, filtered and used directly.

In this laboratory, no correction is made for the volume occupied by the phosphotungstate-protein precipitate and the barium phosphotungstate precipitate. The method has usually been applied to urines containing a small amount of protein. Large bulky precipitates were not encountered. Usually 50 cc of the reagent precipitated the protein, uric acid and creatinine completely and the small excess of reagent did not yield a voluminous precipitate with barium hydroxide solution. Blank determinations were made to ascertain the error present when no correction was made for the volume occupied by the precipitate. Six grams

⁴ Berthold Oppler *Zeitschr f physiol Chem*, lxxv, pp 71-135

of glucose and 0.5 gram of blood serum were dissolved in 200 cc normal urine. Of this solution, 50 cc were defecated by the method as given and of the filtrate from the barium phosphotungstate precipitation, 11.2 cc (average) were required for the complete decolorization of 10 cc Fehling's solution. The corresponding check, a sugar solution containing 1 gram of the same glucose dissolved in 200 cc of water, required 10.55 cc (average) for the complete decolorization of 10 cc of Fehling's solution. At first glance, the error appears large but when one remembers that the original 50 cc has been diluted to 300 cc the error is more than compensated for by the fact that one gets a clean-cut end point in the titration, a thing not encountered except through the use of some defecating agent.

The method is easily carried out and in our hands gives much better solutions for titration than does the use of any other method we have tried. We had the experience of using this method on the defecation of urines containing about 8 per cent of glucose and a good trace of protein. Using the polariscope on the undefecated urine, not eliminating the protein on account of the small amount present, we got only fairly sharp readings, even by using short columns of the liquid for polarization. By the method as outlined we got sharp readings and also higher readings owing to the removal of the laevo rotating protein that cut down the dextro rotation of the mixture. The small amount of protein had a very appreciable effect on the actual percentage of sugar found and on the removal of the protein the glucose reading reached more nearly what corresponded to the actual glucose content of the urine.

The experimental part of this paper was carried out by Mr Charles Coons. The author is indebted to Dr R. E. Lyons for his efforts in overseeing the work during a portion of its progress.

PRELIMINARY NOTE ON A PURINE-HEXOSE COMPOUND

By JOHN A. MANDEL AND EDWARD K. DUNHAM

(From the Chemical Laboratory of the New York University and Bellevue Hospital Medical College, N. Y.)

(Received for publication, January 23, 1912)

From an extract of a commercial preparation of yeast, a crystalline substance was obtained which is a compound of adenine and a hexose. The amount of material isolated has not been sufficient for a complete identification of the sugar, but, pending access to a further supply, a preliminary note on the results obtained at this time appears justified by the interest attaching to such a compound.

The substance separates from aqueous solution in sheaves of delicate, colorless acicular crystals, melting sharply at 206° (uncorrected). It does not give a pentose reaction with orcin and hydrochloric acid. Fehling's solution is not reduced, unless the substance be previously hydrolysed, in that case, cuprous oxide is not precipitated, but a white, flocculent precipitate of a cuprous-purine compound is formed.

A combustion of the substance, dried over sulphuric acid *in vacuo*, yielded the following results:

Weight of substance, 0.1954, CO_2 , 0.3153, H_2O , 0.0930

The nitrogen was determined by the Kjeldahl method.

0.2106 gram of substance yielded ammonia equivalent to 35.8 cc. of decinormal sulphuric acid, and to 23.80 per cent N in the substance.

Phosphorus and sulphur were absent.

A solution of the substance in 1 per cent sulphuric acid is dextrorotatory, the specific rotation being approximately 12.15° . On boiling for an hour in a flask with reflux condenser, the rota-

tion increases and the solution acquires a light yellow color. The addition of picric acid to this solution causes a yellow precipitate, soluble in hot water and separating in crystalline form on cooling. These crystals, after purification, melted at 281° , and yielded 29.23 per cent N, proving this substance to be adenine picrate, which contains 29.37 per cent N.

The filtrate from the picrate precipitate, after removing the picric acid which was present by shaking with ether, yielded a phenylosazone, which could be readily recrystallized from 50 per cent alcohol and melted sharply, without evolution of gas, at 156° . A nitrogen determination gave 15.3 per cent N.

From a portion of the solution containing the hydrolysed substance, some indications were obtained of the formation of laevulinic acid on treatment with strong hydrochloric acid.

The foregoing facts appear to us to indicate unmistakably that the substance isolated by us is a combination of adenine with a hexose. The melting point of the phenylosazone approximates that of the phenylosazone obtainable with sorbose, gulose and idose. We are engaged upon the further identification of the sugar.

A comparison of our analysis with the theoretical composition of an adenine-hexose compound shows a close agreement.

	Found	Calculated for $C_{11}H_{18}N_6O_5$
C	44.08	44.39
H	5.32	5.10
N	23.80	23.61
O	26.80	26.90

The phenylosazone obtained with our substance yielded 15.3 per cent N. A hexosazone contains 15.67 per cent N, while a pentosazone contains 17.1 per cent N.

PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS

FIRST PAPER

BY OTTO FOLIN AND W DENIS

(*From the Biochemical Laboratory of Harvard Medical School, Boston*)

(Received for publication, January 23, 1912)

During the past ten years a great many unsuccessful attempts have been made to find out what becomes of the amino-acids which are formed in the intestine as a result of the digestion of protein. After having worked out new analytical methods which we believed to be more suitable than any previously available for the study of this problem we have now obtained results which seem to throw new light on this phase of protein metabolism.

Besides being hampered by the lack of suitable analytical methods for investigating the non-protein nitrogen of blood, nearly all previous workers have conducted their investigations from a point of view which almost completely eliminated the possibility of accounting for the amino-acids absorbed as such from the digestive tract. The blood has been regarded as essentially a closed system, closed physiologically as well as anatomically, and except for the supposed effective deamidizing power of the liver they have worked on the assumption that the amino-acids absorbed from the intestine should heap up in the blood to such an extent that they could not fail to find them. As a matter of fact the non-protein nitrogen of blood does rise and sink like a tide with reference to absorption from the digestive tract and the variations appear to be adequate to account for all the nitrogen when considered from the right point of view.

An all important function of the blood is to transport food from the digestive tract to every tissue in the body, this being so there is *a priori* no reason why the transport of the amino-acids from the blood to all the various organs should be less prompt than the

transport of those same amino-acids from the digestive tract into the blood

In this connection we may recall the familiar experiment with potassium iodide and the astounding rapidity with which it, when swallowed, is transported to every part of the body. If any one should attempt to account for the potassium iodide absorbed from the digestive tract by determining the amount present in the blood the result would be quantitatively quite as dismal a failure as have been the attempts to account for the amino-acids. But in the absence of positive evidence to the contrary it would seem extremely probable that the amino-acids are distributed in the same manner. At least that is the one possibility which should first of all be eliminated before other explanations for the apparent disappearance of the amino-acids are attempted. The fact that the amino-acids are food materials is only an additional reason for supposing that they are distributed where food materials are needed—which is everywhere.

During the past few years many of us have been under the influence of the concept of intermediary metabolism, of deamidation, urea formation, etc., and have been prone to ascribe to the liver activities and functions without number and without proof. When we began this work we soon discovered that while the liver does almost wholly abstract the ammonia from the portal blood and probably converts it into urea, it does not "deamidize" the amino-acids. It was this discovery which led us to the hypothesis that the blood promptly transports the amino-acids from the intestine to every tissue in the body.

One of the early experiments which we made to prove the correctness of this point of view was to substitute urea for amino-acids. Urea is absorbed from the intestine even more rapidly than amino-acids. There is no reason for believing that it can be lost by a conversion into protein, as has been assumed in the case of the amino-acids, nor is there any reason for believing that the liver would do anything in particular to it, nor that the muscles and other tissues would be more discriminating than the mucous membrane of the small intestine and would fail to absorb it from the blood. By using urea instead of amino-acids we thus eliminated all the various hypotheses which have been advanced to "explain" why the absorbed products can not be found in the blood. The results obtained would seem to be conclusive.

Absorption of Urea

EXPERIMENT 1 Cat 22 (weight 2428 grams) was etherized forty hours after the last meal (meat) A sample of the blood (5 cc) was taken from the right carotid artery and another (5 cc) from the portal vein The blood supply to both kidneys was cut off by ligatures to prevent the urea from escaping The right iliac artery was also ligatured and an outside ligature was applied to the whole leg to prevent the urea from getting into it by way of the lymph The small intestine was ligatured just below the stomach and just above the caecum One hundred cubic centimeters of warm 4 per cent urea solution was then injected into the intestine by means of a large syringe Forty-five minutes after the urea injection we again took samples of blood from the portal vein and from the left carotid artery We then washed out the urea solution remaining in the intestine and finally cut out similar pieces of muscle from each of the two hind legs to be used for urea determinations

The analyses gave the following figures

	<i>Milligrams</i>
Total urea nitrogen injected	1866
Total urea nitrogen recovered from intestine	430
Total urea nitrogen absorbed	1436
Total non-protein nitrogen per 100 cc of portal blood before the urea injection.	38
Total urea nitrogen in the same	23
Total non-protein nitrogen per 100 cc of carotid blood before the injection.	38
Total urea nitrogen in the same	23
Total non-protein nitrogen per 100 cc of portal blood after the urea injection	154
Total urea nitrogen in the same	122
Total non-protein nitrogen per 100 cc of carotid blood after the injection	138
Total urea nitrogen in the same	92
Total non-protein nitrogen per 100 grams of muscle from the ligatured leg	190
Total urea nitrogen in the same	50
Total non-protein nitrogen per 100 grams muscle of the normal leg	220
Total urea nitrogen in the same	94

This being our first attempt to apply the analytical methods to muscle the absorbed values may not be correct The compara-

tive aspect, the difference found, should, however, be nearly correct for the two muscles were treated exactly alike. We probably used too much substance to get out all the non-protein nitrogen.

The following experiment is rather more illuminating as a study of the absorption of urea.

EXPERIMENT 2 Cat 25 (weight 2513 grams) This cat had received a large amount of meat about twenty-four hours before the experiment. The stomach was found empty but the intestine still contained some food in the process of digestion. After etherization a sample of blood was drawn from the portal vein. The arteries and veins of the kidneys were then ligatured. The gracilis muscle was removed from the left hind leg and at once prepared for analysis. The small intestine was tied off as in Experiment 1 and 4 grams of urea in about 100 cc of warm water was injected. Small samples of blood (2 cc) were drawn at different intervals and the total non-protein nitrogen ("n p nitrogen") and the urea nitrogen determined.¹

At the end of the experiment the intestine was washed out with warm water and the washings saved for analysis. The right gracilis muscle was also removed and analyzed for total non-protein nitrogen and for urea nitrogen.

	<i>Milligrams</i>
Total urea nitrogen injected	1866
Total urea nitrogen recovered	811
Total soluble nitrogen recovered	943
Hence urea nitrogen absorbed	1055
Total n p nitrogen per 100 cc of portal blood before the urea injection	37
Urea nitrogen in the same	28
Total n p nitrogen per 100 cc of carotid blood two and one-half minutes after the injection	50
Urea nitrogen in the same	35
Total n p nitrogen per 100 cc of portal blood five and one-half minutes after the injection	67
Urea nitrogen in the same	57
Total n p nitrogen per 100 cc of carotid blood twelve minutes after the injection	85
Urea nitrogen in the same	77

¹ In drawing the portal blood the needle was inserted by way of one of the tributary veins which could afterwards be clamped without obstructing the flow of blood through the portal vein.

	<i>Milligrams</i>
Total n p nitrogen per 100 cc of portal blood in thirteen minutes after the injection	102
Urea nitrogen of the same	95
Total n p nitrogen per 100 cc of arterial blood (femoral) thirty minutes after the injection	92
Urea nitrogen in the same	80
Total n p nitrogen per 100 cc of mesenteric vein blood thirty-one minutes after the injection	117
Urea nitrogen in the same	95
Total n p nitrogen per 100 cc of portal blood fifty-five minutes after the injection.	127
Urea nitrogen in the same	108
Total n p nitrogen per 100 grams of muscle before the urea injection	273
Urea nitrogen in the same	31
Total n p nitrogen per 100 grams of muscle about one hour after the urea injection	342
Urea nitrogen in the same	86

Before the two experiments recorded above had been made we had satisfied ourselves by a number of preliminary experiments with pancreatic digestion mixtures that their absorption from the small intestine is accompanied by unmistakable increase of the non-protein nitrogen in the blood. The following one with glycocoll proves beyond reasonable doubt that this amino-acid is absorbed from the intestine in much the same way as urea (though less rapidly) and that it is rapidly absorbed from the blood by the tissues.

Absorption of Glycocoll

EXPERIMENT 3 Cat 26 (weight 2143 grams) was etherized about forty hours after the last meal which consisted of a little (50 grams) meat. Two cubic centimeters of blood were drawn from the portal vein. The arteries and veins of both kidneys were then ligatured as in Experiment 1. The iliac artery of the right hind leg was clamped while the gracilis muscle was removed. The circulation through the leg was then restored by removing the clamp from the artery. The small intestine was tied off as in Experiments 1 and 2, and 10 grams of glycocoll in about 100 cc of warm water were injected. Small samples of blood (2 cc) were drawn at the end of 6, 18, and 45 minutes.

At the end of the experiment the gracilis muscle was removed from the other leg. The analyses gave the following results

	<i>Milligrams</i>
Total glycocoll nitrogen injected	1867
Total nitrogen recovered from the intestine	1300
Hence, glycocoll nitrogen absorbed	567
Total non-protein nitrogen per 100 cc of portal blood before the injection	30
Total urea nitrogen in the same	18
Total n p nitrogen per 100 cc of portal blood six minutes after the injection	36
Urea nitrogen in the same	20
Total n p nitrogen per 100 cc of carotid blood six minutes after the injection	34
Urea nitrogen in the same	19
Total n p nitrogen per 100 cc of portal blood eighteen minutes after the injection	55
Urea nitrogen in the same	22
Total n p nitrogen per 100 cc of carotid blood eighteen minutes after the injection	47
Urea nitrogen in the same	22
Total n p nitrogen per 100 cc of mesenteric blood forty-five minutes after the injection	85
Urea nitrogen in the same	21
Total n p nitrogen per 100 cc of carotid blood forty-five minutes after the injection ²	57
Urea nitrogen in the same	21
Total n p nitrogen per 100 grams of muscle taken before the experiment	250
Urea nitrogen in the same	27
Total n p nitrogen per 100 grams of muscle at the end of the experiment	346
Urea nitrogen in the same	27

In the above experiment it will be noted that the non-protein nitrogen in the portal blood at any given time is considerably higher than the non-protein nitrogen in the general systemic blood. This might suggest deamidation, but the figures for the urea show that it is not a case of deamidation and urea formation. It is simply a question of amino-acid absorption due to the constantly increasing concentration of glycocoll in the portal blood. The

² A preliminary sample of about 3 cc was drawn from the carotid in this case, and was thrown away so as to get rid of the blood which had remained confined in the artery since the last withdrawal.

idea that the amino-acid nitrogen should be converted into urea by the intestine and liver as fast as absorbed is after all not entirely satisfactory, for it fails to indicate how that deamidation is regulated so as not to deprive the various tissues of nitrogenous material, some of which at least must be needed. The condition revealed by the above experiment with glycocholate on the other hand shows that the amino-acid nitrogen is not immediately split off and converted into urea. On the contrary in this experiment we have failed to find any urea formation.

So far as the urea formation is concerned the following experiment is rather interesting

Absorption of Pancreatic Digestion Mixture

EXPERIMENT 4 Cat 18 (weight 1785 grams) was etherized twenty-four hours after the last meal (consisting of 40 grams of chopped meat). After taking the samples of normal blood the intestine was ligatured and 97 cc of the warm digestion mixture introduced. This mixture of self-digested beef pancreas was over two years old and gave no biuret reaction. It was slightly alkaline to litmus and 20 per cent of its total nitrogen consisted of ammonia.

	<i>Milligrams</i>
Total amino-acid nitrogen injected	737
Total nitrogen recovered at the end of seventy minutes	410
Hence amino-acid nitrogen absorbed	327
Total n p nitrogen per 100 cc of portal blood before the injection	40
Urea nitrogen in the same	24
Total n p nitrogen per 100 cc of carotid blood before the injection	38
Urea nitrogen in the same	24
Total n p nitrogen per 100 cc of portal blood fifteen minutes after the injection	54
Urea (and ammonia) nitrogen in the same	34
Total n p nitrogen per 100 cc of portal blood sixty-three minutes after the injection	64
Urea and ammonia nitrogen in the same	38
Total n p nitrogen per 100 cc of carotid blood sixty minutes after the injection	66
Urea nitrogen in the same	34

Whether all the urea increase found is due to the preformed ammonia or whether some of it is due to deamidation is as yet undetermined. In this particular experiment we did not determine the ammonia in the blood but we had done this in previous experiments and found that the ammonia in the portal blood rose to about seven times the normal value under the influence of about the same amount of the digestion mixture as was used in Experiment 3.

When egg albumin is used instead of amino-acids the result obtained is different.

Absorption of Egg Albumin

EXPERIMENT 5 Cat 23 (weight 643 grams) was etherized and 61 grams of warm white of egg was injected into the ligatured intestine. The following results were obtained

	<i>Milligrams</i>
Total nitrogen injected as egg white	915
Total nitrogen recovered	850
Hence, total nitrogen absorbed	65
Total n p nitrogen per 100 cc of portal blood before the injection	36
Urea nitrogen in the same	22
Total n p nitrogen per 100 cc of carotid blood before the injection	36
Urea nitrogen of the same	24
Total n p nitrogen one and one-half hours after the injection in 100 cc of portal blood	42
Urea nitrogen in the same	20
Total n p nitrogen per 100 cc of carotid blood one and a half hours after the injection	42
Urea nitrogen in the same	20

In addition to the detailed experiments described above we wish to record the following summary (p 95) of the non-protein nitrogen and of urea nitrogen in blood as found under more ordinary conditions.

In the above pages we have confined ourselves to the presentation of analytical results which seem to show what becomes of the amino-acids absorbed from the intestinal tract. The muscles and other tissues as well evidently serve as a storehouse for such reserve materials. The existence of such a reservoir must be

Total non-protein nitrogen and urea nitrogen in normal blood in milligrams per 100 cc

	PORTAL BLOOD		SYSTEMIC BLOOD	
	Total N	Urea-N	Total N	Urea-N
Cat 10 fasting			40	23
Cat 18 fasting	40	24	38	24
Cat 20 fasting (pregnant)			29	16
Cat 22 fasting	38	23	38	23
Cat 23 fasting	36	24	36	22
Cat 16 fed meat	40	24	38	24
Cat 19 fed much meat			54	34
Cat 21 fed sugar and cream	26	19	26	19
Cat 24 fed sugar and cream	30	14		
Fresh slaughter house blood (beef)			24	17

taken into account in our theories of protein metabolism, for it certainly ought to make at least some points clear which were not clear before. The peculiar lag extending over several days in the establishment of a constant level of nitrogen elimination when extreme changes are made in the nitrogen intake is probably due to a filling or a depletion as the case may be of the reservoir. The different results obtained when a single substance like creatine or an amino-acid is fed together with diets rich or poor in nitrogen would also be determined by the condition of the reservoir. When full the creatine is eliminated and the amino-acid augments the urea output, when nearly empty both are retained. We hope to report more experimental data on this subject very soon. We are continuing our investigations on the absorption and distribution of nitrogenous materials (food products and waste products) and we hope that we may be allowed to reserve for a little while the new field which we believe has been opened by this research.

The analytical methods used are adaptations of colorimetric methods for the determination of nitrogen, urea and ammonia in urine. None of these have as yet been published in detail, though some of them have been given privately to a number of persons. All will be published in full very soon.

HYDANTOINS THE ACTION OF POTASSIUM THIOCYANATE ON ALANINE

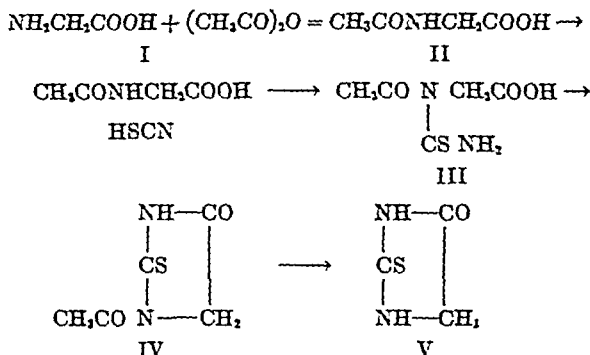
NINTH PAPER

By TREAT B JOHNSON

(From the Sheffield Laboratory of Yale University)

(Received for publication, January 18, 1912)

In a recent publication from this laboratory, Johnson and Nicolet¹ have described a synthesis of 2-thiohydantoin (V) They prepared this compound by the action of potassium thiocyanate on amino-acetic acid (I) in the presence of acetic anhydride and acetic acid, and showed that the general reactions involved in the condensation are to be represented as follows

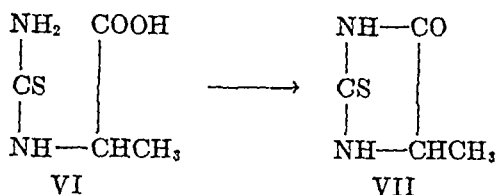


In other words, the product of the reaction is 2-thio-3-acetylhydantoin (IV), which can be converted quantitatively into the 2-thiohydantoin (V), by hydrolysis with concentrated hydrochloric acid This method of synthesizing 2-thiohydantoin was also described by Komatsu,² but his interpretation of the mechanism of the reaction was entirely incorrect Komatsu also exam-

¹ *Journ Amer Chem Soc*, xxviii, p 1974

² *Memoirs Coll Sci and Eng, Kyoto University, (Japan)*, iii, p 1

ined the behavior of potassium thiocyanate towards alanine and states that this amino-acid reacts with the rhodanide in the presence of acetic anhydride, giving the corresponding thiohydantoic acid (VI), which is converted into 2-thio-4-methylhydantoin (VII), by digestion with hydrochloric acid



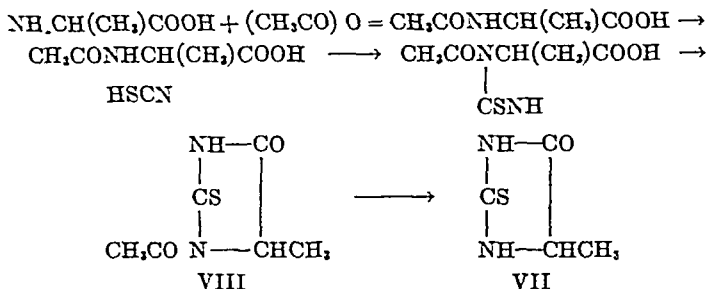
It seemed very probable to the writer that glycocoll and alanine would react in a similar manner with potassium thiocyanate, under the conditions employed by Komatsu, giving the corresponding acetylated thiohydantoins. However, it was not improbable that these two acids might behave in a different manner and that in the case of alanine Komatsu actually was dealing with a thiohydantoic acid derivative. The analytical results obtained by analysis of the barium salt of his acid, however, do not offer strong evidence that Komatsu's compound possessed the structure which he assigned to it. He found 29.6, 29.5 and 30.3 per cents of barium and concluded therefore that he was dealing with a hydrous barium salt, which theoretically contains 30.73 per cent of barium. He did not determine the percentage of water of crystallization.

I now find that alanine reacts smoothly with potassium thiocyanate, in the presence of acetic anhydride, forming 2-thio-3-acetyl-4-methylhydantoin (VIII). In fact, I obtained no evidence of the formation of a thiohydantoic acid (VI) as described by Komatsu. The constitution of my acetyl compound was established not only by the analytical determinations, but also by the fact that the same compound was formed when acetylalanine³ was used for the synthesis instead of alanine. When the acetylthiohydantoin (VIII) was digested with hydrochloric acid it was converted quantitatively into 2-thio-4-methylhydantoin VII. This hydantoin has previously been prepared in this laboratory.⁴

³ Fischer and Otto *Berichte*, *lvi*, p. 2114

⁴ Wheeler, Nicolet and Johnson *Amer Chem Journ*, *lvi*, p. 456

by a different method. Komatsu⁵ showed that this thiohydantoin can be desulphurized by mercury oxide giving 4-methylhydantoin. Alanine therefore reacts with potassium thiocyanate in a similar manner as glycocoll and the chemical changes involved are to be represented as follows

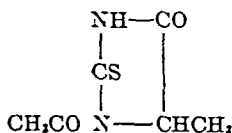


These nitrogen-unsubstituted 2-thiohydantoins are representatives of a new class of hydantoins. A knowledge of their chemical properties is especially desirable since it is probable that such cyclic groupings may be involved in the molecular structure of sulphur proteins. Like the thiopolyptides they contain the thioamide grouping, $-\text{CSNH}-$, and as the writer has previously indicated,⁶ thioamides probably functionate in the natural synthesis of sulphur proteins from simpler substances. We shall continue our investigation of this interesting class of sulphur compounds and will discuss in future publications their biological significance.

EXPERIMENTAL PART

The Action of Potassium Thiocyanate on Alanine, $\text{NH}_2\text{CH}(\text{CH}_3)\text{COOH}$

2-Thio-3-acetyl-4-methylhydantoin



⁵ *Loc cit*

⁶ *This Journal*, 17, p 331

This thiohydantoin can be prepared easily in the following manner. Dissolve 2 grams of alanine and 2 grams of finely pulverized, dry potassium thiocyanate in a mixture of 9 cc of Kahlbaum's acetic anhydride and 1 cc of glacial acetic acid by warming in a water-bath. Connect the flask with a return condenser in order to avoid the absorption of moisture from the air. On warming, there is an immediate reaction and within two minutes a clear yellow solution is obtained. The liquid is then heated, at 100°, for about twenty-five minutes, cooled and then poured into about five volumes of cold water when the greater proportion of the above acetyl hydantoin will separate in a crystalline condition. The yield is about 2.5 to 2.7 grams and in every experiment tried the substance melted, without further purification, at 163 to 165° to a clear oil. The hydantoin crystallizes from 95 per cent alcohol in stout prisms, which melt at 166° to an oil. On cooling, the oil solidified in the capillary tube and on heating again it melted at 166° as before.

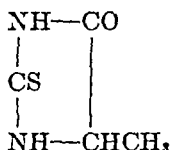
ANALYSIS Sulphur determination (Carius)

0.1377 gram substance gave 0.1939 gram BaSO₄

Nitrogen determination (Kjeldahl)

	Calculated for C ₆ H ₇ O ₂ N S	Found
N	16.27	16.33
S	18.8	19.30

Hydrolysis of the Acetylthiohydantoin with Hydrochloric Acid
2-Thio-4-methylhydantoin



The acetyl derivative was suspended in about ten to fifteen parts of concentrated hydrochloric acid and the mixture warmed on the steam-bath. The hydantoin completely dissolved and after evaporation of the acid practically a quantitative yield of this hydantoin was obtained. The substance was purified for analysis by recrystallization from hot 95 per cent alcohol. It separated, on cooling, in beautiful, hexagonal tables or plates which melted at 161° to a clear oil.

ANALYSIS (Kjeldahl)

N

Calculated for $C_6H_5ON_2S$	Found
21.53	21.7

The hydantoin was identical with the hydantoin obtained from alanine by Wheeler, Nicolet and Johnson ⁷

The Formation of 2-Thio-3-acetyl-4-methylhydantoin from Acetylalanine, $CH_3CO NHCH(CH_3)COOH$ ⁸

The acetylalanine was warmed with the required proportion of potassium thiocyanate under the same conditions as when alanine was used. After heating one-half hour to complete the reaction, and finally cooling, the liquid was then poured into cold water. A yellow solid separated at once and after purification by crystallization from alcohol it melted at 165 to 166°. A mixture of this substance with the above acetyl hydantoin, prepared from alanine, melted at exactly the same temperature.

⁷ *Loc cit*

⁸ Fischer and Otto *Loc cit*

FASTING STUDIES VI

DISTRIBUTION OF NITROGEN DURING A FAST OF ONE HUNDRED AND SEVENTEEN DAYS

By PAUL E. HOWE, H. A. MATTILL AND P. B. HAWK

(From the Laboratory of Physiological Chemistry of the University of Illinois)

(Received for publication, January 13, 1912)

If the literature of fasting be searched it will be found that the records indicate that adult dogs may live for periods ranging from thirty days to fifty days without partaking of food provided free access to water is permitted. At the time the data embraced in the present paper were reported¹ the longest *normal* fast on record so far as we are aware, was that reported by Falck² in which the dog used as subject fasted sixty days. The ninety-eight-day fast reported by Kumagawa and Miura³ cannot be considered as a *normal* fast, inasmuch as the animal was subjected to the influence of phlorhizin.

The reports of fasting tests in which human beings have served as subjects afford data on authentic fasts ranging in length from two to fifty days. The most complete data on short fasts have been furnished by Benedict.⁴ Of the longer fasts those on Beauté,⁵

¹ Howe, Mattill and Hawk. Boston Meeting, Soc. Biol. Chem., Dec., 1909, *Proceedings Soc. Biol. Chem.*, July, 1910.

² Falck. *Beitr. Physiol.* (Stuttgart), 1875. Quoted by Pashutin in *Pathological Physiology*, 1902.

³ Kumagawa and Miura. *Arch. f. Physiol. u. Anat. (physiol. Abt.)*, p. 431, 1898.

⁴ Benedict. Carnegie Pub. 77 (1907).

⁵ Cathcart. *Biochem. Zeitschr.*, vi, p. 199, 1907.

Tosca,⁶ Schenck,⁷ Succi,⁸ Cetti,⁹ Breithaupt,¹⁰ "E" and "H,"¹¹ Tanner,¹² and Merlatti¹³ are the most important. The longest of these are the thirty-day fasts of Succi, the forty-day fast of Tanner and the fifty-day fast of Merlatti.

DESCRIPTION, PLAN, ETC

The methods of analysis employed in our investigation were the same as those used in fasting studies already reported from this laboratory.¹⁴ In conformity with the custom in this laboratory in experiments of this character the dog used as subject was not catheterized but was allowed to urinate at will. This explains to a degree the cause of the irregularity in the urine volumes which obtains in the early part of the experiment, notwithstanding the fact that the urine was measured at a uniform time from day to day. We prefer this mode of urine collection to the catheterization procedure because of the attendant danger of infecting the animal during the latter process.

The diet used in the preliminary period of the experiment was as follows:

<i>Constituent</i>	<i>Grams</i>
Meat	400
Cracker dust	100
Lard	45
Bone ash	12
Water	700

⁶ Van Hoogenhuyze and Verploegh *Zeitschr f physiol Chem*, xlv1, p 415, 1905-06

⁷ Brugsch and Hirsch *Zeitschr f exp Path u Therap*, iii, p 638, 1906

⁸ Luciani *Das Hungern*, Leipzig, 1890, Ajello and Solaro *La riforma medica*, xv, 2, p 542, 1893, E and O Freund *Wiener klin Rundschau*, xv, pp 69 and 91, 1901

⁹ Lehman, Muller, Munk, Senator, Zuntz *Virchow's Archiv*, cxvii, suppl., 1893

¹⁰ Id, *ibid*

¹¹ Howe, Mattill and Hawk *Jour Amer Chem Soc*, xxxiii, p 568, 1911

¹² Lusk *Science of Nutrition*, 2d Ed, p 55, 1909

¹³ Merlatti *Luciani's Das Hungern*, 1890

¹⁴ Howe and Hawk *Jour Amer Chem Soc*, xxxiii, p 215, 1911

The above diet contained 15.796 grams of nitrogen. Approximate nitrogen equilibrium was secured after an eight-day feeding interval. The balance for this period was as follows:

	<i>Income</i>	<i>Grams</i>
Food		15.796
	<i>Outgo</i>	
Feces		0.372
Hair		0.359
Cage washings		0.147
Urine		15.588
		<hr/>
		-16.466
		+15.796
		<hr/>
		-0.670

The subject of the experiment was our fasting dog "Oscar," an adult Scotch collie weighing 26.33 kg. at the opening of the fast. Inasmuch as his preliminary diet contained 15.796 grams of nitrogen per day he was receiving about 3.75 grams of protein per kilogram of body weight during the preliminary period.

It was our intent at the start of the investigation to fast the animal to the pre-mortal rise in nitrogen excretion, then to bring the dog back to the normal condition by means of careful feeding and subsequently to fast him a second time. In other words we wished to make a study of "repeated fasting" similar to the one already reported from this laboratory.¹⁵

DISCUSSION OF RESULTS

It was evident from the very beginning of the fasting period that "Oscar" was not being influenced by the fasting régime in as pronounced a manner as were other fasting dogs in adjoining cages. There was a less rapid loss of weight, a less precipitate destruction of body tissues as shown by the nitrogen output and a conservation of bodily vigor and energy not noted in the case of any of the other dogs. As time passed each of the associated dogs in succession reached the "pre-mortal rise" in nitrogen excretion. At the

¹⁵ Howe and Hawk. *Loc. cit.*

forty-eighth day, when the experiment upon the initial fast of the last dog terminated, "Oscar" was so full of vigor that he jumped into his cage from the floor. In performing this act it was necessary for him to project his body upward to a height of about three feet. He had been in the habit of jumping into his cage each day after being weighed but the practice was discontinued after the fifty-eighth fasting day in order to protect the dog from possible injury due to coming in contact with the sharp corners of the cage front. He continued to jump out of his cage up to and including the one hundred and first day of the fast. It was apparently quite a task for him at this time in his weakened condition to maintain his equilibrium after leaping from his cage to the floor. In order to avoid injury, he was, therefore, not permitted to perform this feat after the one hundred and first fasting day. The animal, however, continued to wag his tail vigorously and frequently barked when we approached his cage, all of which seemed to indicate that he was in "good spirits" even up to the very end of the experiment. It is an interesting fact that one of our dogs was fasted to the pre-mortal rise, then subjected to an intermediate equilibrium feeding period during which time the dog regained its original body weight and then fasted a second time to the pre-mortal rise while "Oscar" was undergoing his initial fast and jumping in and out of his cage daily, and no sign of the pre-mortal rise being apparent.

When the one hundred and seventeenth day was reached it was decided to terminate the fast. The dog now weighed 9.76 kg as against 26.33 kg at the opening of the fast, a loss of about 63 per cent in body weight. It was then June 2 and the animal had been fasting continuously since February 6. At the commencement of the experiment we had expected to be able to finish two fasts and the intermediate feeding period in the four months which had passed. The great length of the first fast had nullified this arrangement. It was therefore decided to initiate the second fast at the opening of the next college year.

During the summer the dog passed the time on a Kansas farm under close observation. He was brought back in the fall and upon examination was found to weigh somewhat more than he did at the commencement of his first fast. He also seemed to be stronger, more energetic and in better all round physical condition.

than he had been before he was subjected to the one hundred and seventeen-day fast. After being brought into nitrogen equilibrium he was then subjected to a second fast. The data from this "repeated fast" will be presented in a subsequent paper.¹⁶

Distribution of Nitrogen

The general data for each individual day of the one hundred and twenty-five days of the experiment are given in Table I, pp 108-111. To facilitate discussion and comparison the data have been placed in Table II, p 112 in the form of four-day averages. The course of the excretion of various forms of nitrogen has also been represented in graphic form in Fig I, p 115. The percentage values are given in Table III, p 113.

TOTAL NITROGEN If we examine Table II, p 112, it will be seen that the average daily output of nitrogen during the eight-day feeding period was 15.588 grams. This value was lowered to 6.231 grams for the first four-day fasting interval whereas the three succeeding four-day periods showed progressively decreasing nitrogen values, the figures being 4.471 grams, 4.028 grams and 3.216 grams respectively. From this point the output of nitrogen fluctuated irregularly until the twenty-first period, or eighty-first day of the fast at which time a more uniform level was assumed and fairly well maintained throughout the remainder of the fast.

The slight rise in the nitrogen excretion upon the last day of the fast cannot be considered as the beginning of the pre-mortal rise. In the first place the pre-mortal rise is invariably preceded by a slight decrease in the nitrogen excretion, a condition not observed in this experiment. A much more potent argument against considering the slight increase in the nitrogen of the final fasting day as an indication that the pre-mortal rise had been established is found in the fact that at no time had the daily output of creatine-nitrogen exceeded that of creatinine-nitrogen. In all the fasting studies made in this laboratory, where the animals have fasted to the pre-mortal rise, we have noted in every instance that the output of creatine increases during the final stages of the fast and finally a few days before the fall in the nitrogen output which

¹⁶ Reported before American Physiological Society, Baltimore, December, 1911.

108 Elimination of Nitrogen During Fasting

TABLE I
General Data

DAY OF EXPERIMENT	BODY WEIGHT	VOLUME OF URINE	SPECIFIC GRAVITY	TOTAL N	UREA N	AMMONIA N	CREATININE N	CREATINE N	PURINE N	ALLANTOIN N
<i>Preliminary Period—700 cc water per day</i>										
	<i>kgs</i>	<i>cc</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
1		474	10135	10 504	8 795	0 479	0 271			
2		983	1026	17 354	14 908	0 608	0 421	0 470		0 031
3		448	1018	6 050	5 165	0 390	0 187		0 050	
4		1330	1028	24 770	21 477	0 948	0 666	0 484	0 056	0 066
5	26 36	400	1030	9 900	7 881	0 341	0 193	0 236		
6	26 36	360	1031	8 920	7 698	0 330	0 311	0 108		
7	26 36	1028	1028	23 192	20 479	1 000	0 622	0 331	0 069	0 025
8	26 33	1043	1028	24 136	21 070	0 986	0 608	0 430	0 095	0 032
Average				15 588	13 434	0 635	0 410	0 343	0 065	0 035

Fasting Period—700 cc water per day

1	25 82	680	1015	9 326	8 051	0 394	0 373	0 109	0 098	
2	25 34	380	10125	2 029	1 421	0 244	0 091	0 024		
3	25 05	276	1016	4 532	3 715	0 324	0 186	0 092		
4	24 69	885	10125	9 038	7 323	0 630	0 536	0 162	0 035	0 065
5	24 37	496	10115	4 734	3 800	0 355	0 289	0 083	0 020	0 025
6		590	1009	4 820		0 416	0 322	0 112		
7		337	10105	3 072	2 428	0 241	0 185	0 054	0 013	0 021
8	23 80	455	10125	5 260	4 312	0 335	0 275	0 107	0 021	0 028
9		200	1013	2 364	1 963	0 102	0 132	0 046		
10	23 42	449	1014	6 161	4 969	0 400	0 357	0 058	0 024	0 021
11	22 98	219	10235	4 284	3 477	0 257	0 221	0 021		
12	22 90	520	1008	3 320	2 672	0 242	0 171	0 042		
13	22 89	980	10055	4 099	3 179	0 357	0 329	0 032	0 018	0 014
14	22 54	637	1003	1 657	1 341	0 115	0 097	0 003	0 010	0 007
15	22 35	318	1011	2 432	1 934	0 198	0 138	0 010	0 006	0 100
16		660	10095	4 678	3 722	0 384	0 242	0 055	0 013	0 025
17	22 12	588	10065	3 444	2 805	0 217	0 220	0 009	0 020	0 008
18	21 83	790	1008	5 966	4 860	0 368	0 310	0 058	0 023	
19	21 57	350	1003	1 269	1 075	0 071	0 078	0 00	0 004	0 003
20	21 49	422	1007	2 970	2 407	0 222	0 191	0 00		
21	21 14	725	10085	5 420	4 440	0 346	0 309	0 004		
22	21 03	529	1005	2 520	2 132	0 156	0 172	0 00		
23	20 82	470	1006	2 650	2 188	0 189	0 163	0 00		
24	20 75	600	10085	4 825	3 888	0 325	0 271	0 041		
25	20 61	500	1003	1 933	1 631	0 115	0 123	0 00	0 139	0 019
26	20 27	700	10085	5 219	4 306	0 305	0 327	0 00		
27	20 10	457	10085	3 518	2 892	0 184	0 224	0 016		
28	20 00	476	1007	3 225	2 553	0 254	0 206	0 00		
29	19 96	460	1004	1 715	1 379	0 131	0 115	0 00		

TABLE I—(Continued)

DAY OF X-IR RIMFNT	BODY WEIGHT	VOLUME OF URINE	SPECIFIC GRAVITY	TOTAL N	UREA N	AMMONIA N	CREATININE N	CHLORINE N	PURINE N	ALLANTOIN N
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Fasting Period—700 cc water per day—Continued

	kgs	cc		grams	grams	grams	grams	grams	grams	grams
30	19 84	505	10070							
31	19 73	460	1006	5 817	4 738	0 347	0 389	0 00		
32	19 52	504	10075							
33	19 31	600	1010	6 325	6 768	0 587	0 557	0 00		
34	19 10	300	1002							
35	19 03	472	1007	4 125	3 305	0 258	0 274	0 00	0 114	0 066
36	18 82	435	1006							
37	18 69	660	1007	6 307	5 165	0 390	0 393	0 00		
38	18 49	500	1007							
39	18 47	428	1004	5 557	4 439	0 399	0 395	0 00		
40	18 26	640	1006							
41	17 98	652	1004	7 278	5 978	0 453	0 507	0 00		
42	17 87	550	10045							
43	17 87	425	10035	4 744	3 917	0 292	0 310	0 004		
44	17 79	520	10035							
45	17 39	720	1005	5 986	4 905	0 366	0 359	0 016	0 181	0 065
46	17 27	300	10040							
47	17 16	450	1007	4 866	4 072	0 317	0 139	0 030		
48	17 00	610	1009							
49	16 99	484	1003	6 488	5 307	0 456	0 383			
50	16 86	485	1008							
51	16 62	555	1007	7 317	5 917	0 567	0 420	0 010		
52	16 56	530	1006							
53	16 44	455	1007	6 246	5 668	0 498	0 306	0 00		
54	16 34	485	1005							
55	16 21	500	10045	5 207	4 233	0 446	0 291	0 00	0 153	0 111
56	15 99	550	10075							
57	15 95	460	10075	6 739	5 985	0 666	0 306	0 00		
58	15 90	450	1005							
59	15 78	500	1005	5 285	4 506	0 452	0 305	0 00		

Water increased to 2100 cc per day

60	16 09	1385	1002	5 097	4 054	0 469	0 223	0 034	0 005	0 036
61	15 79	2390	1002	3 690	2 980	0 362	0 174	0 071	0 004	0 024
62	15 59	1680	1001	3 096	2 430	0 301	0 142	0 032	0 002	0 036
63	15 32	1840	1002	2 793	2 203	0 284	0 138	0 045	0 002	0 025

700 cc water per day

64	14 94	820	10035	2 256	1 796	0 224	0 118	0 032	0 002	0 016
65	14 71	640	1004	3 016	2 421	0 268	0 159	0 022	0 012	0 018
66	14 64	545	1005	2 173	1 790	0 192	0 107	0 021	0 007	0 010

TABLE I—(Continued)

DAY OF EXPERIMENT	BODY WEIGHT	VOLUME OF URINE	SPECIFIC GRAVITY	TOTAL N	UREA N	AMMONIA N	CREATININE N	CREATINE N	PURINE N	ALLANTOIN N
<i>700 cc water per day—Continued</i>										
	<i>kgs</i>	<i>cc</i>		<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
67	14 44	625	10045	3 566	2 862	0 329	0 163	0 00	0 131	0 030
68	14 14	192	1019	2 997	2 446	0 249	0 135	0 00		
69	14 20	180	10135	2 580	2 136	0 218	0 117	0 00		
70	14 21	480	10035	1 805	1 480	0 146	0 070	0 004		
71	13 98	740	10055	3 947	3 179	0 352	0 148	0 047	0 036	0 031
72	13 87	600	1006	3 573	2 885	0 309	0 134	0 019		
73	13 70	612	10035	2 571	2 089	0 230	0 106	0 012		
74	13 61	573	10045	3 266	2 671	0 275	0 120	0 013		
75	13 62	428	1004	4 113	3 544	0 156	0 073	0 016	0 048	0 027
76	13 38	675	1006	3 942	3 207	0 323	0 144	0 028		
77	13 27	565	10045	3 024	2 488	0 242	0 111	0 025		
78	13 60	568	10045	2 609	2 173	0 212	0 089	0 025		
79	13 00	630	1005	3 588	2 932	0 307	0 119	0 039	0 037	0 040
80	13 02	367	1004	1 854	1 548	0 136	0 060	0 025		
81	13 07	398	1005	2 278	1 885	0 176	0 081	0 012		
82	12 80	711	1004	3 842	3 156	0 303	0 140	0 015		
83	12 65	602	10035	3 436	2 841	0 278	0 121	0 013	0 047	0 048
84	12 65	475	1004	2 002	1 672	0 153	0 072	0 015		
85	12 46	655	10065	3 578	3 017	0 261	0 109			
86	12 37	550	10055	2 784	1 822	0 469	0 092	0 013		
87	12 43	479	1003	1 956	1 603	0 163	0 066	0 021	0 034	0 065
88	12 20	680	10035	3 534	2 911	0 253	0 119	0 061		
89	12 26	448	10025	1 841	1 541	0 119	0 062	0 034		
90	12 14	587	10035	2 931	2 458	0 188	0 091	0 043		
91	11 97	670	1003	2 891	2 427	0 200	0 094	0 035	0 034	0 065
92	12 02	465	10025	1 950	1 638	0 132	0 062	0 017		
93	11 78	630	1003	3 186	2 703	0 216	0 105	0 020		
94	11 78	530	10025	2 080	1 750	0 141	0 068	0 012		
95	11 65	620	1003	2 928	2 559	0 171	0 090	0 027	0 034	0 065
96	11 52	557	1003	2 582	2 209	0 153	0 079	0 032		
97	11 52	486	10015	1 930	1 601	0 125	0 052	0 029		
98	11 50	467	1002	2 124	1 862	0 131	0 057	0 032		
99	11 32	597	1004	3 308	2 816	0 195	0 039	0 037	0 034	0 065
100	11 19	550	1003	2 079		0 152	0 060	0 027		
101	11 18	412	10025	1 983		0 132	0 059	0 021		
102	11 11	490	10025	1 853		0 144	0 052	0 028		
103	11 00	573	10025	2 207		0 181	0 061	0 031	0 034	0 065
104	10 92	540	10025	1 822		0 148	0 055	0 023		
105	10 85	579	1002	2 530	2 181	0 181	0 065	0 038		
106	10 79	563	10015	2 316	1 793	0 307	0 055	0 030		
107	10 82	472	10015	1 724	1 307	0 242	0 043	0 024	0 034	0 065
108	10 64	556	1003	2 878	2 247	0 336	0 065	0 039		

*Big loss in weight and low urine volume due to fact that no water was given the dog on sixty-seventh day

TABLE I—(Concluded)

DAY OF EXPERIMENT	BODY WEIGHT	VOLUME OF URINE	SPECIFIC GRAVITY	TOTAL N	UREA N	AMMONIA N	CREATININE N	CREATINE N	PURINE N	ALLANTOIN N
700 cc water per day—Continued										
	kgs	cc		grams	grams	grams	grams	grams	grams	grams
109	10 59	575	1002	2 263	1 760	0 330	0 055	0 030		
110	10 48	622	1002	2 385	2 014	0 173	0 058	0 030		
111	10 38	601	1003	2 444	2 088	0 173	0 055	0 043		
112	10 18	635	1003	2 665	2 255	0 198	0 056	0 043		
113	10 23	473	1002.5	2 244	1 915	0 138	0 046	0 044		
114	10 15	510	11.015	2 211	1 883	0 140	0 048	0 041		
115	10 05	514	1002.5	2 368	2 303	0 163	0 049	0 028		
116	10 02	550	1002	2 390	2 035	0 106	0 047	0 052		
117	9 76	536	1003	2 780	2 371	0 174	0 046	0 042		

precedes the pre-mortal rise the *creatinine-nitrogen excretion is found to be greater than that of creatinine-nitrogen*¹⁷ If the data for the creatine-nitrogen and creatinine-nitrogen excretion of the present experiment be examined it will be noted that the creatine-nitrogen output at no time exceeded that of creatinine-nitrogen This fact precludes any possibility of considering the slightly increased nitrogen output of the last fasting day as the beginning of the pre-mortal rise in the nitrogen excretion

The pronounced increase in the nitrogen output for the sixteenth period was due to the fact that the daily water ration was increased from 700 cc to 2100 cc for each of the days of this period The influence of this high water ingestion has already been discussed by us in another connection¹⁸ The conclusion drawn from this increased nitrogen excretion when taken into connection with the creatine, purine, and allantoin data hereinafter discussed was to the effect that the high water ingestion had caused increased protein catabolism This augmented output of nitrogen is neatly represented in Fig I, p 115

UREA-NITROGEN For the most part the urea excretion ran closely parallel with that of total nitrogen This fact is especially

¹⁷ Howe and Hawk *Loc cit*, Howe, Mattill and Hawk *Proceedings Amer Soc Biol Chem*, July, 1910

¹⁸ Howe, Mattill and Hawk *This Journal*, v, p 417, 1911

TABLE II
Nitrogen Distribution—Four-day Averages

FOUR DAY PERIOD	TOTAL N	UREA N	AMMONIA N	CREATININE N	CREATINE N	PURINE N	ALLANTOIN N	UNDETERMINED N
	grams	grams	grams	grams	grams	grams	grams	grams
Normal (8 days)	15 588	13 434	0 635	0 410	0 343	0 065	0 035	0 666
1	6 231	5 102	0 121	0 296	0 097	0 062	0 031	0 522
2	4 471	3 513	0 337	0 268	0 089	0 018	0 024	0 222
3	4 028	3 270	0 400	0 220	0 042	0 024	0 021	0 051
4	3 216	2 544	0 263	0 201	0 025	0 012	0 038	0 133
5	3 410	2 787	0 219	0 199	0 017	0 017	0 004	0 169
6	3 854	3 162	0 254	0 226	0 012	0 013	0 002	0 185
7	3 474	2 845	0 214	0 220	0 004	0 013	0 002	0 176
8*	3 172	2 577	0 213	0 212	0 000	0 013	0 007	0 150
9	2 608	2 130	0 162	0 167	0 000	0 013	0 007	0 129
10	3 209	2 604	0 213	0 225	0 000	0 015	0 007	0 145
11	2 682	2 205	0 164	0 175	0 005	0 018	0 006	0 109
12	2 838	2 357	0 193	0 132	0 008	0 018	0 006	0 124
13	3 391	2 896	0 266	0 169	0 003	0 015	0 011	0 031
14	2 983	2 555	0 274	0 172	0 000	0 015	0 011	
15†	2 642	2 253	0 226	0 152	0 000	0 015	0 011	
16‡	3 669	2 917	0 376	0 169	0 046	0 003	0 031	0 127
17	2 752	2 217	0 253	0 139	0 019	0 010	0 012	0 102
18	2 832	2 310	0 241	0 118	0 012	0 026	0 006	0 119
19	3 380	2 797	0 242	0 108	0 015	0 007	0 006	0 205
20	3 291	2 700	0 271	0 115	0 029	0 009	0 006	0 161
21	2 852	2 357	0 223	0 100	0 017	0 008	0 007	0 140
22	2 580	2 028	0 261	0 085	0 016	0 008	0 009	0 173
23	2 799	2 334	0 145	0 091	0 043	0 009	0 010	0 167
24	2 536	2 163	0 165	0 081	0 019	0 007	0 012	0 089
25	2 486	2 122	0 151	0 069	0 032			0 112
26	2 120		0 155	0 058	0 027			
27	2 123	1 760	0 219	0 055	0 029			0 060
28	2 492	2 022	0 253	0 058	0 036			0 123
29	2 372	2 014	0 160	0 049	0 039			0 160
30§	2 390	2 038	0 166	0 047	0 032			0 107
31§	2 780	2 371	0 174	0 046	0 042			0 147

* Average for five days

† Average for two days

‡ Period of high water ingestion

§ Single days

TABLE III
Percentage Nitrogen Distribution

FOUR DAY PERIOD	UREA N	AMMONIA N	CREATININE N	CREATINE N	PURINE N	ALANTOIN N	UNDETERMINED N
<i>Preliminary Period</i>							
8 Day Average	per cent 86 11	per cent 4 07	per cent 2 63	per cent 2 20	per cent 0 42	per cent 0 22	per cent 4 27
<i>Fasting Period</i>							
1	81 89	1 94	4 75	1 56	1 00	0 50	8 38
2	78 59	7 54	6 00	1 99	0 40	0 54	4 97
3	81 16	9 93	5 46	1 04	0 60	0 52	1 27
4	79 09	8 18	6 25	0 78	0 37	1 18	4 14
5	81 69	6 42	5 85	0 50	0 50	0 12	4 95
6	82 05	6 59	5 86	0 31	0 38	0 05	4 80
7	81 91	6 16	6 33	0 12	0 37	0 06	5 07
8*	81 25	6 72	6 68	0 00	0 41	0 22	4 73
9	81 66	6 21	6 40	0 00	0 50	0 27	4 95
10	81 14	6 64	7 01	0 00	0 47	0 22	4 52
11	82 20	6 11	6 52	0 19	0 67	0 22	4 06
12	83 14	6 80	4 65	0 28	0 63	0 21	4 37
13	85 40	7 84	4 98	0 09	0 44	0 32	0 91
14	85 57	9 31	5 76	0 00	0 50	0 37	
15†	85 28	8 55	5 75	0 00	0 57	0 42	
16†	79 49	10 25	4 61	1 25	0 08	0 84	3 46
17	80 57	9 19	5 05	0 69	0 36	0 44	3 71
18	81 57	8 51	4 17	0 42	0 92	0 21	4 20
19	82 74	7 16	3 19	0 44	0 21	0 18	6 06
20	82 05	8 24	3 50	0 88	0 27	0 18	4 89
21	82 64	7 82	3 51	0 60	0 28	0 25	4 91
22	78 61	10 11	3 29	0 62	0 31	0 35	6 71
23	83 39	5 18	3 25	1 54	0 32	0 36	5 97
24	85 29	6 51	3 19	0 75	0 28	0 47	3 51
25	85 37	6 07	2 78	1 29			4 51
26		7 31	2 74	1 27			4 48
27	83 21	10 31	2 59	1 37			2 83
28	81 14	10 15	2 33	1 44			4 93
29	84 91	6 75	2 07	1 64			4 64
30§	85 27	6 95	1 97	1 34			4 48
31§	85 28	6 26	1 65	1 51			5 29

Average for five days.
† Average for two days.‡ Period of high water ingestion
§ Single day

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TABLE IV

Body Weights, Creatinine Coefficients, Urine Volumes and Water Balance

FOUR DAY PERIOD	BODY WEIGHT END OF FOURTH DAY	AVERAGE LOSS IN BODY WEIGHT PER DAY	LOSS IN BODY WEIGHT PER DAY	CREATININE FOUR DAY AVERAGE	CREATININE COEFFICIENT	URINE VOLUME FOUR DAY AVERAGE	WATER INGESTION PER DAY	WATER EXCRETION
<i>Preliminary Period</i>								
8 Day Average	kgs 26 33	kgs	per cent	grams 0 410	15 6	cc 708	cc 700	per cent 101 2
<i>Fasting Period</i>								
1	24 69	0 41	1 56	0 296	12 0	556	700	79 5
2	23 80	0 22	0 84	0 268	11 2	470	700	67 1
3	22 90	0 23	0 87	0 220	9 6	347	700	49 6
4	22 35	0 14	0 53	0 201	9 0	649	700	92 7
5	21 49	0 22	0 84	0 199	9 3	538	700	76 9
6	20 75	0 19	0 72	0 226	10 9	581	700	83 0
7	20 00	0 14	0 53	0 220	11 0	533	700	76 1
8*	19 31	0 14	0 53	0 212	11 0	506	700	72 3
9	18 69	0 16	0 61	0 167	8 9	467	700	66 7
10	17 98	0 18	0 68	0 225	12 5	578	700	82 6
11	17 39	0 15	0 57	0 175	10 1	554	700	79 1
12	16 99	0 10	0 38	0 132	7 8	470	700	67 1
13	16 44	0 14	0 53	0 169	10 3	521	700	74 4
14	15 98	0 12	0 46	0 172	10 8	449	700	64 1
15†	15 78	0 10	0 38	0 152	9 6	475	700	67 9
16‡	15 32	0 46	1 75	0 169	11 0	1825	2100	86 9
17	14 44	0 22	0 84	0 139	9 6	657	700	93 9
18	13 98	0 12	0 46	0 118	8 5	491	700	70 1
19	13 62	0 09	0 34	0 108	7 9	544	700	77 7
20	13 00	0 16	0 61	0 115	8 8	609	700	87 0
21	12 65	0 09	0 34	0 110	8 7	519	700	74 1
22	12 43	0 06	0 23	0 085	6 8	540	700	77 1
23	11 97	0 12	0 46	0 091	7 6	596	700	85 1
24	11 65	0 08	0 30	0 081	7 0	561	700	80 1
25	11 32	0 08	0 30	0 069	6 1	527	700	75 3
26	11 00	0 08	0 30	0 058	5 3	497	700	71 0
27	10 82	0 05	0 19	0 055	5 1	540	700	77 1
28	10 38	0 11	0 42	0 058	5 6	581	700	83 0
29	10 05	0 08	0 30	0 049	4 9	533	700	76 1
30§	10 02	0 03	0 11	0 047	4 7	550	700	78 6
31§	9 76	0 26	0 99	0 046	4 7	596	700	83 7

* Average for five days

† Average for two days

‡ Period of high water ingestion

§ Single day

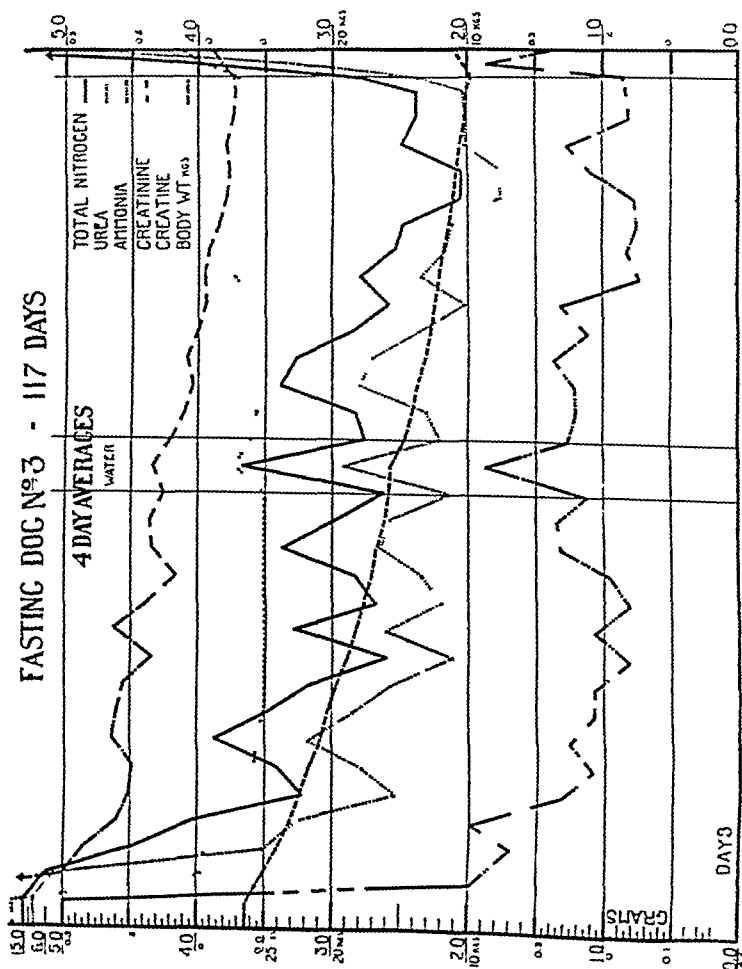


Figure 1

clearly shown in the figure. Some interesting relationships are observed when the data for the percentage of the total nitrogen output which was excreted in the form of urea (Table III, p 113) are examined. During the feeding period a trifle more than 86 per cent of the total nitrogen had been excreted as urea-nitrogen. Under the influence of the fasting metabolism this value decreased to an average of 81.5 per cent for an interval of forty-eight days. At this point in the fast the urea values rose to a value somewhat above 85 per cent for twelve days, then sank to the low level for about a month, finally coming back to the 85 per cent level for the final portion of the fast. Our data therefore show in general a decreased output of nitrogen in the form of urea during the fast above that excreted during the period of normal feeding. However, these data do not substantiate the claim of numerous investigators¹⁹ that the percentage output of urea *decreases as the fast progresses*. In this experiment the region of low urea values was during the first part of the fast whereas the region of high values occurred during the more advanced stages of fasting. We have verified the truth of the claim mentioned above in connection with certain experiments on fasting men²⁰ in which a gradually decreasing percentage output of urea-nitrogen was observed from the beginning to the end of the fasting interval. However, in the case of fasting dogs we have never succeeded in demonstrating a similar course for the urea output.²¹

While it is true that the urea values during the first part of the fast were somewhat lower than at later stages of the test, it will be observed that the variation was not marked. The urea values may be looked upon, therefore, as more or less *uniform* in the case of fasting dogs. On the other hand in the case of fasting men there is a marked *decrease* in the percentage output of urea as the fast progresses, the decrease in one of our experiments being from 89.6 per cent to 79.2 per cent in six days, and in another instance the drop being from 86.2 per cent to 75.8 per cent in a similar interval.

¹⁹ E and O Freund *Loc cit*, Brugsch *Zeitschr f exp Path u Therap*, 1 and III, 1906, Osterberg and Wolf *Biochem Zeitschr*, v, p 304, 1907, Underhill and Kleiner *This Journal*, iv, p 165, 1908, Schondorf *Pflüger's Archiv*, cxvii, p 257, 1907, Cathcart *Loc cit*.

²⁰ Howe, Mattill and Hawk *Journ Amer Chem Soc*, xxxiii, p 568, 1911.

²¹ Howe and Hawk *Loc cit*.

The reason for the difference in the course of the urea output of fasting dogs as compared with fasting men may be, as expressed in another paper from this laboratory,²² in the fact that the dog, whether normally nourished or fasting is deriving its energy primarily in each instance, from nutritive material of the same character, *i e*, *fresh lean meat* when normally fed and *muscular tissue* when fasted. On the other hand man is accustomed to a cooked diet of a much lower protein content than the dog consumes and his cells are therefore confronted by very unusual conditions when asked to catabolize body tissue as they must perforce do in the course of a fasting test. It is not at all beyond the realm of possibility that the differences just mentioned may account at least in part for the fact that the course of the fasting excretion of urea-nitrogen is different in the organism of the dog from that observed in the human organism.

AMMONIA-NITROGEN In common with the values for total nitrogen and urea-nitrogen the excretion of nitrogen in the form of ammonia underwent a sharp decline at the opening of the fast from the value as determined for the period of normal feeding. The actual figures for the average daily output were 0.635 gram for the feeding interval as against 0.121 gram for the first four-day period of the fast. The values for the two succeeding four-day periods were 0.337 gram and 0.400 gram respectively, but from this point for an interval of about two and one-half months the excretion of ammonia-nitrogen was fairly uniform. There were low values for the ninth and eleventh periods and a very high value for the sixteenth period (water) but apart from these variations the general level of the excretion was about 0.25 gram. At this time (twenty-third period) there came an abrupt decrease in the ammonia values the data indicating an average daily output of about 0.160 gram for the remaining periods of the fast with two exceptions.

The high ammonia value mentioned as occurring in the sixteenth period was due to the influence of the increased quantity of water fed the animal during each of the days of that period. It will be remembered that the usual daily allowance of 700 cc. was increased to 2100 cc. on each of these four days. The increased output of ammonia we interpret as an index of stimulated gastric function.

This feature of the fast has been discussed in another connection²³ and the interpretation offered is right in line with other interpretations from this laboratory which have had to do with the influence of water upon the ammonia excretion of the normally nourished individual²⁴

CREATININE-NITROGEN During the period of normal feeding the average daily output of creatinine-nitrogen was 0.410 gram. This value underwent a gradual decrease as the fast progressed, as is indicated by an output of 0.296 gram during the first period, one of 0.152 gram during the fifteenth period about two months later and one of 0.046 gram upon the one hundred and seventeenth day. This downward tendency of the creatinine output may be followed very nicely in Fig. I, p. 115. It is also of interest to note that the body weight curve as plotted in this figure runs in general parallel with the curve representing the creatinine output. In other words as the body lost in weight the creatinine output decreased. These facts are in line with the claim that the creatinine output is a function of the amount of active muscular tissue in the body²⁵. We have made similar observations in connection with other fasting studies²⁶.

If Table I be examined it will be observed that the output of creatinine-nitrogen for the sixtieth day was far above the average output for the experiment up to that time. This was the day upon which the water ingestion of the animal was increased 200 per cent above the usual quota, as before mentioned. In experiments previously made in this laboratory in which men and animals have been subjected to the influence of fasting or water drinking the creatinine output has without exception been *decreased* under these conditions. It is interesting, therefore, in the present instance where we have the influence of the two factors (fasting and water drinking) exerted *simultaneously* upon the same individual, that the creatinine output should be *increased* rather than decreased. This feature has been more fully discussed in a previous paper to which reference has already been made.

²³ Howe, Mattill and Hawk. *This Journal*, x, p. 417, 1911.

²⁴ Fowler and Hawk. *Journ. Exp. Med.*, xii, p. 388, 1910; Wills and Hawk. *Proceedings Amer. Soc. Biol. Chem.*, *This Journal*, ix, p. 244, 1911.

²⁵ Folin. *Amer. Journ. of Physiol.*, xvii, p. 66, 1905; Shaffer. *Ibid.*, xvi, p. 252, 1906; McCollum. *Amer. Journ. of Physiol.*, xiv, p. 210, 1911.

²⁶ Howe and Hawk. *Loc. cit.*; Howe, Mattill and Hawk. *Loc. cit.*

At the very end of the fast it will be noted by examining Fig I, p 115, that the curves for the excretion of nitrogen in the forms of creatinine and creatine approach rather closely to each other *but do not cross*. The relation of this fact to the absence of any pre-mortal rise in the nitrogen excretion is discussed elsewhere in this paper in connection with the excretion of creatine and total nitrogen.

The percentage of the total nitrogen which was excreted as creatinine (Table III, p 113) decreased gradually as the fast progressed.

CREATINE-NITROGEN During the course of the eight-day preliminary period there was a daily average of 0.343 gram of creatine-nitrogen excreted by the dog. This creatine excretion is of course directly traceable to the fact that the dog is a "high protein" animal. As we have already said, the diet of the dog in question contained 3.75 grams of protein per kilogram of body weight. In other words a man of 70 kg weight if fed on the same level would be ingesting over 260 grams of protein per day. This is a protein ingestion about two and one-half times greater than that suggested by the Voit standard²⁷ and about five times greater than that suggested by Chittenden.²⁸ The normal human organism does not excrete ingested creatine to any degree unless such an organism be living upon a high protein level similar to that above mentioned.²⁹ The gradual increase in the creatine output accompanying an increase in the diet is very nicely shown in the study of "repeated fasting" recently reported from this laboratory by Howe and Hawk.

In the present instance upon the first day of the fast there was, of course, a very sharp drop in the creatine elimination, only 0.109 gram being eliminated whereas the average daily output for the first four-day period was 0.097 gram as against a daily average of 0.343 gram for the interval of high protein feeding. From this point the creatine excretion underwent a gradual decrease until the eighth period at which time the urine of the dog was found to be practically *creatinine-free*. This period of low creatine values continued for nearly one month or until the end of the fifteenth period.

²⁷ Lusk's *Science of Nutrition*, 2d Ed., 1909.

²⁸ Chittenden *Physiological Economy in Nutrition*, 1904.

²⁹ Folin *Hammarsten Festschrift*, p 15, 1906.

At the opening of the sixteenth period it will be observed that creatine again appeared in the urine in large quantities, the excretion of this constituent being greater than at any time in the whole experiment subsequent to the second period or eighth day. In other words in a total of one hundred and nine fasting days the period in question showed the highest creatine values. This is all the more striking when we recall the fact that it follows immediately after an interval during which the urine was to all intents and purposes creatine-free.

By referring to Fig. I, p. 115, the course of the creatine-nitrogen excretion may be very conveniently followed. It will be seen that the curve gradually descends during the early part of the fast and in the eighth period, after about one month's fasting, it assumes the low level mentioned, and continues at this low level until the opening of the sixteenth period as before mentioned. This period of very low creatine values is represented on the figure by a virtually straight line thus accentuating the following rise of the sixteenth period.

It will be remembered that this sixteenth period was the interval during which the daily water quota of the animal was increased from the usual one of 700 cc. to one three times as great, i. e., 2100 cc. In previous work from this laboratory³⁰ upon the influence of water drinking upon the creatine excretion it has been demonstrated that the ingestion of large volumes of water by normally nourished men was followed by the appearance of creatine in the urine. The creatine data of the experiments mentioned have been offered as the first *direct* experimental evidence in support of the hypothesis that the increased nitrogen output which follows water drinking is due to a *stimulation of protein catabolism* and not to a simple *flushing of the tissues*. Bearing these findings in mind the high creatine values of the water period of this fasting study are very significant. Here we have an animal which has been fasting for nearly two months, receiving a daily ingestion of water amounting to 700 cc. Under these conditions the urine volumes averaged about 500 cc. indicating that the tissues and organs of the dog must have been pretty well flushed during each day of the fasting interval. Moreover the urine was practically

³⁰ Fowler and Hawk. *Loc. cit.*, Howe and Hawk. Unpublished.

creatinine-free as has already been mentioned. At this very opportune moment of minimum creatinine values the water ingestion of the animal was increased 200 per cent, and coincident with this increased water intake comes the augmented creatinine output. Certainly it is perfectly logical to conclude in this connection that the water was the active factor in bringing about the increase in the quantity of creatinine eliminated. We very naturally look to the muscular tissue when we inquire as to the origin of the creatinine. The water has evidently been instrumental in causing a true catabolism of protein material. As discussed in a previous paper,³¹ however, when we attempt to show a definite relationship between the total nitrogen figures and those for creatinine-nitrogen our calculations indicate a discrepancy. The total nitrogen output was increased 3.188 grams during the water period, a nitrogen quota equivalent to 98 grams of flesh if we take the value 3.25 per cent for the nitrogen percentage of flesh. Taking creatinine in a similar way and using 0.123 per cent as the creatinine-nitrogen value of flesh we find that the increased creatinine-nitrogen of the water period aggregated 0.182 gram, a value equivalent to 148 grams of flesh. There is thus a discrepancy of 34 per cent between our total nitrogen and our creatinine-nitrogen figures if we consider that each type of value represents the complete disintegration of muscular tissue. This being true, we were forced to the conclusion, as already discussed elsewhere, that *creatinine may be removed from muscular tissue and excreted in the urine without its removal of necessity being accompanied by the complete disintegration of that tissue*. In support of this contention we would offer certain other evidence obtained in connection with fasting experiments carried out in this laboratory.³² In these tests the creatinine content of muscle was much decreased as the result of fasting, a decrease of over 60 per cent being noted. The nitrogen content of this same muscle was however but slightly lowered. This low creatinine value for a muscle which still retains its original nitrogen quota practically unaltered is a very significant finding, and emphasizes again the inaccuracy of considering the *total* amount of creatinine excreted as having arisen from the complete and permanent disintegration of muscular tissue. It is evident then that *creatinine may be removed*

³¹ Howe, Mattill and Hawk. *This Journal*, x, p. 417, 1911.

³² Howe and Hawk. *Journ. Amer. Chem. Soc.*, xxxii, p. 215, 1911.

from tissues which are still functioning within the body Mendel and Rose³³ have recently reported an *increase* in the creatine content of the muscles of fasting rabbits and hens In this connection they have objected to certain of our interpretations The matter has received further consideration from us in a recent article³⁴

After leaving the water period the curve for the excretion of creatine-nitrogen descends abruptly and from this low plane begins a somewhat gradual rise to the end of the fast Coincidentally with this rise in the creatine-nitrogen curve it will be noted that the curve representing the output of creatinine-nitrogen descends They approach very close to each other *but do not cross* This fact is of great significance when taken into consideration with other creatine and creatinine data collected by us in recent fasting studies In every instance in which our animals have been fasted to the so-called pre-mortal rise in the nitrogen excretion, the creatinine-nitrogen output has *decreased* during the later stages of the fasting interval and this decrease has been associated with a much more pronounced *increase* in the output of creatine-nitrogen When these values were plotted it was noticed that the curves for the excretion of creatine and creatinine, in every case, *crossed a few days before the fall in the nitrogen output which preceded the pre-mortal rise* This "creatine crossing" occurred with great regularity at practically the same point with respect to this fall in total nitrogen output and it is believed to be a sign of more than ordinary significance It will be studied further in this laboratory On the basis of our knowledge regarding the relationship between the "creatine crossing" and the ultimate death of an animal we estimate that "Oscar" would have been able to live at least one hundred and thirty days without food

When the elimination of total nitrogen was discussed in a previous paragraph attention was called to the fact that there was a slight increase in the nitrogen output upon the one hundred and seventeenth or final day of the fast This slight increase in the nitrogen excretion is not believed to be connected in any way with the pre-mortal rise in nitrogen excretion inasmuch as it was preceded neither by a decreased output of nitrogen nor by the phenomenon we have termed the "creatine crossing"

³³ Mendel and Rose This *Journal*, v, p 255, 1911

³⁴ Howe, Mattill and Hawk This *Journal*, v, p 417, 1911

An examination of Table III, p. 113, will show that there was not only an actual increase in the output of creatine-nitrogen during the final stages of the fast but also an accompanying increased percentage output as well. In other words the increased output of creatine was accompanied by a less pronounced increase or by a decrease in the output of total nitrogen.

PURINE-NITROGEN These values and those for allantoin-nitrogen were determined for the first ninety-six days of the one hundred and seventeen-day fast, and reported in connection with other data on the allantoin and purine output of fasting dogs³⁵. The data are included in the tables of the present paper in order that the records may be complete on this exceptionally long fast. A brief summary of the findings in this connection will be given at this time. The purine values were somewhat irregular during the fast but there was nevertheless a decided tendency toward a *decreased* output as the fast progressed. For example if we compute the average output for the first half of the fast and compare this with the average output for the second half of the fast we observe that the output was considerably *decreased* during the second half of the fast. Scaffidi³⁶ has recently reported a decreased output of purine-nitrogen by a dog during the course of a sixteen-day fast. On the other hand Schittenhelm,³⁷ and Underhill and Klemer³⁸ found the course of the excretion to be irregular. In the interesting work of Hunter and Givens³⁹ on the purine excretion of the coyote the course of the elimination of this form of nitrogen was not studied inasmuch as composite urine samples were utilized for analysis.

By referring to the data for the water period in Tables I and II, pp. 109 and 112, it will be observed that the purine-nitrogen output *decreased* in a very marked manner during the time of high water ingestion. This phenomenon is discussed later.

ALLANTOIN-NITROGEN The output of allantoin-nitrogen during the fast was irregular. However, if we compare the output for the first thirty days of the fast with the output for the last thirty

³⁵ Wreath and Hawk, *Journ. Amer. Chem. Soc.*, **xxviii**, p. 1601, 1911.

³⁶ Scaffidi, *Biochem. Zeitschr.*, **xxviii**, p. 153, 1911.

³⁷ Schittenhelm, *Zeitschr. f. physiol. Chem.*, **lxii**, p. 80, 1909.

³⁸ Underhill and Klemer, *This Journal*, **iv**, p. 165, 1908.

³⁹ Hunter and Givens, *This Journal*, **viii**, p. 449, 1910.

days we find that this output has decreased 40 per cent, *i e*, from 0.4 gram to 0.24 gram. None of the previous investigators of the fasting output of allantoin have observed such a marked decrease during the final stages of the fast as we have recorded here.

Upon the days of high water ingestion the output of allantoin was *increased* in a very pronounced manner. Previous to this water period the average daily excretion of allantoin-nitrogen had been 0.011 gram. The high water intake caused this daily value to be increased more than three-fold on the first day of its ingestion, the values remaining quite uniformly high throughout the period. It will be recalled that the values for the purine-nitrogen excretion were *decreased* during this interval of copious water ingestion in which the allantoin-nitrogen values were *increased*. Furthermore it has been shown by Rulon and Hawk⁴⁰ that the uric acid output is *decreased* under the influence of an increased water ingestion. It is well known that purine bodies may be oxidized to allantoin and furthermore that the allantoin excretion of an animal may be increased by purine feeding. It therefore seems fair to conclude that the large volume of water introduced into the body of this fasting dog has markedly stimulated the oxidation mechanism and consequently such substances as would under ordinary conditions go to augment the purine-nitrogen output have been transformed into allantoin and are excreted in this form.

If we consider the total output of nitrogen of purine origin (purine-nitrogen + allantoin-nitrogen) we observe that it is increased during the interval of high water intake. This may be taken as further evidence in the support of the hypothesis that at least a part of the increase in the total nitrogen output observed to follow copious water drinking is due to a true protein catabolism rather than to a flushing of the tissues. As before mentioned in connection with the discussion of the output of creatine, the increased nitrogen excretion during the water period was equivalent to 98 grams of flesh. If we calculate the purine-nitrogen value⁴¹ for this 98 grams we find it is 0.059 gram whereas the actual increase in this form of nitrogen was but 0.032 gram. In other words, we cannot account for 46 per cent of the theoretical quantity of

⁴⁰ Rulon and Hawk *Journ Amer Chem Soc*, **XXII**, p 1686, 1910

⁴¹ Hall *The Purine Bodies in Foodstuffs*, Manchester, 1902, p 29, Table

purine-nitrogen. The cause of this discrepancy may be due partly to the method of analysis employed and partly to the fact that the allantoin was further oxidized with the resultant formation of other nitrogenous substances.

UNDETERMINED NITROGEN The data for the actual output of this form of nitrogen indicate considerable irregularity as would naturally be expected in a fasting organism. In general the values were higher during the first half of the fast than they were during the later stages of the fasting interval. When we consider the percentage output of undetermined nitrogen as given in Table III, p. 113, we observe a much greater uniformity from period to period than is ordinarily the case. The average daily percentage value was about 4.6 per cent which was a trifle higher than the value for the period of normal feeding preceding the fast, i. e., 4.3 per cent. Other fasting tests already reported from this laboratory⁴² have also shown very uniform undetermined nitrogen values for fasting periods. In the instances cited, however, the level of the feeding periods was considerably above the fasting level, whereas in the present experiment this variation was not noted.

Body Weights, Creatinine Coefficients, Urine Volumes and Percentage Water Elimination

The dog weighed 26.33 kg. at the start of the fast whereas his weight at the end of the fast on the one hundred and seventeenth day was 9.76 kg. He had lost approximately 63 per cent in body weight. The daily loss was greater during the first portion of the fast than during the later stages, as would logically be expected. There was in general a gradual decrease in the daily loss in weight up to the time the water ingestion of the animal was increased from 700 cc. to 2100 cc. This high water intake occurred during the sixteenth period. If Table IV, p. 114, be examined it will be observed that the average daily loss for over a month previous to this time had been 0.10 to 0.15 kg. With the advent of this interval of copious water ingestion, however, the daily loss increased to 0.46 kg. this being the highest daily loss sustained by the animal at any time during the fast. The loss in weight was still marked (0.22 kg.) during the period following the time of high water intake.

⁴² Howe and Hawk, *Journ. Amer. Chem. Soc.*, **xxiii**, p. 215, 1911.

but from that time up to the last day of the fast the average daily loss was in general progressively decreased. The loss upon the one hundred and seventeenth fasting day was 0.26 kg. This big loss was due partly to a high urine volume and a mild diarrhoea.

The creatinine-coefficient was 15.6 for the preliminary feeding period. At the opening of the fast the coefficient dropped to 12.0 and decreased slowly and irregularly from this point to the eighteenth period. For the remainder of the fast the coefficient decreased rather more sharply, the fast ending with a coefficient of 4.7.

The average daily urine volume during the preliminary period was 708 cc, a volume which was practically identical to the daily water ingestion. As the fast opened the urine flow very naturally fell somewhat maintaining an average of about 500 cc for the interval up to the time of high water ingestion, i. e., the sixteenth period. The average urine volume for the later part of the fast was somewhat higher than it had been at earlier stages in the fast, the value being about 550 cc as against 500 cc. The volumes were more uniform from day to day and from period to period in the later portion of the fast. With few exceptions, however, the daily urine volumes showed a satisfactory uniformity throughout the fast when we take into consideration the fact that the animal was not catheterized. The urine was acid in reaction throughout the fast.

Differential leucocyte counts were made throughout the fast, a report of the findings having already been presented.⁴³

SUMMARY

The subject of the fast was a Scotch collie dog ("Oscar") weighing 26.33 kg at the opening of the fast. The fast was one hundred and seventeen days in length thus constituting by many days the longest fast on record. The dog gave evidence of being possessed of wonderful vigor and stamina. This was indicated by the fact that he was able to jump out of his cage so late as the one hundred and first fasting day.

At the end of the fast of one hundred and seventeen days the animal was carefully fed and ultimately brought back to his original

⁴³ Howe and Hawk. *Proc. Am. Soc. Biol. Chem.*, 1911.

body weight and subjected to a second fast. The data from this second fast will soon be published.

The urine of the animal was examined quantitatively for total nitrogen, urea, ammonia, creatinine, creatine, allantoin and purine-nitrogen. The total nitrogen content of the feces was also determined.

During the pre-fasting interval, the dog was fed a diet containing 3.75 grams of protein per kilogram body weight. He also received 700 cc of water per day during the feeding interval as well as during the fast.

The body weight loss aggregated about 63 per cent for the one hundred and seventeen day fast, the actual weight being 26.33 kg. before the fast and 9.76 kg. on the one hundred and seventeenth day.

There was no indication of a pre-mortal rise in the nitrogen excretion. The "creatinine crossing," *i. e.*, the point in a fast at which the output of nitrogen in the form of creatine exceeds that in the form of creatinine, was not in evidence. This fact is interpreted as indicating that the dog would probably have been able to fast a total of at least one hundred and thirty days if he had not been fed upon the one hundred and seventeenth day.

At the end of the fifty-ninth fasting day, the water ingestion of the dog was raised to 2100 cc per day for an interval of four days. This caused an increase of 77.5 per cent in the total nitrogen output for the first day, urea, ammonia, creatinine, creatine, and allantoin being simultaneously increased, whereas purine was decreased in quantity.

The creatinine coefficient was 15.6 for the period of normal feeding preceding the fast, 12.0 at the opening of the fast and 4.7 on the one hundredth and seventeenth fasting day.

The percentage nitrogen distribution was in general similar to that reported by us in connection with shorter fasts on dogs.

STUDIES ON WATER DRINKING XIII

(FASTING STUDIES VIII)

HYDROGEN ION CONCENTRATION OF FECES¹

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INTRODUCTION

The reaction of feces has been determined qualitatively by v Koziczkowski² who noted the color change of litmus in aqueous extracts of feces Hemmeter³ also observed the reaction in fecal extracts and noted the varying reactions of the same extract to different indicators The use of various indicators for the determination of the degree of acidity of the intestinal contents has been reported by Mattes,⁴ Macfadyen, Nencki and Sieber⁵ and also by Hemmeter,⁶ and the results show that the true reaction varies slightly, but is in general not far from neutral

The results of the authors already mentioned as having made a study of the reaction of feces with the use of various indicators and of v Oefele,⁷ Schmidt and Strasburger⁸ and Lynch⁹ show the re-

¹ Reported before the American Society of Biological Chemists at Baltimore, Dec , 1911

² v Koziczkowski *Deutsch med Woch* , 1904, No 33

³ Hemmeter *Arch f d ges Physiol* , lxxx, p 151, 1900

⁴ Mattes *Berliner klin Woch* , 1898, p 539 (XVI Kongress für Innere Medizin, Wiesbaden)

⁵ Macfadyen, Nencki and Sieber *Arch f exp Path u Pharm* , xxviii, p 311, 1891

⁶ Hemmeter *Loc cit*

⁷ v Oefele *Statistische Vergleichstabellen zur pract Koprologie*, Jena, 1904 (from Schmidt and Strasburger)

⁸ Schmidt and Strasburger *Die Faeces des Menschen*, Berlin, 2d Edition, 1905

⁹ Lynch *Copologia*, Tesis, Buenos Aires (from Schmidt and Strasburger, 1896, p 52)

action of normal feces to be approximately neutral, i e, acid to indicators which give changes in color at hydrogen ion concentrations of 1×10^{-7} or less and alkaline to indicators changing color at hydrogen ion concentrations greater than that value. Similar conditions have been observed by us in our work upon the reaction and hydrogen ion concentration of feces, in which we used methyl orange, litmus, lacmoid, rosolic acid and phenolphthalein papers to test the reaction. Methods for the determination of the titratable acidity of feces have been proposed by Rubner,¹⁰ who extracted with water and titrated with baryta water, by Blauberg,¹¹ Boas,¹² and J. Muller¹³ who titrated with $\frac{N}{10}$ sodium hydroxide (hydrochloric or sulphuric acids for alkaline stools) using phenolphthalein or litmus paper as an indicator. Langstein¹⁴ observed that the titratable acidity depended upon the indicator used.

The neutral, slightly alkaline or amphoteric (Lynch,¹⁵ Schmidt and Strasburger,¹⁶ Hecht,¹⁷ Nothnagel¹⁸) reaction observed in the normal feces of an individual on a mixed diet, may give place to a distinctly acid or alkaline reaction, according to the kind of food ingested. The reactions of the normal stools as shown by the use of different indicators have been explained for the neutral stools as due to the presence of carbonates, and perhaps other gas-forming substances, and phosphates (Mattes,¹⁹ Hemmeter²⁰). For acid stools the presence of large quantities of fatty acids has been suggested which may result from excessive carbohydrate fermentation or from the poor utilization of the ingested fat. The

¹⁰ Rubner *Zeitschr f Biol*, xv, p 159, 1879

¹¹ Blauberg *Experimentelle und kritische studien uber Säuglings Faeces*, Berlin, 1896, p 42

¹² Boas *Diagnostik und Therapie der Darmkrankheiten*, Leipzig, 1898, p 103

¹³ J. Muller *Über die Reaktion der normalen Säuglings Faeces*, Diss Rostock, 1907

¹⁴ Langstein *Jahrbuch für Kinderheilkunde*, lvi, p 330 (cited by Hecht)

¹⁵ Lynch *Loc cit*

¹⁶ Schmidt and Strasburger *Loc cit*, p 106

¹⁷ Hecht *Die Faeces des Säuglings und des Kindes*, Berlin, 1910 p 20

¹⁸ Nothnagel *Beitrage zur Physiol und Path des Darms*, Berlin 1884, p 79

¹⁹ Mattes *Loc cit*

²⁰ Hemmeter *Loc cit*, p 156

alkaline stools which accompany pronounced putrefaction are due largely to the resulting ammonia (Schmidt and Strasburger)²¹

Especial attention has been given to the reaction of infant stools and the data show that with the ingestion of mother's milk an acid stool results, whereas with the ingestion of cow's milk the reaction is alkaline (Blauberg,²² Hellstrom,²³ Langstein,²⁴ Schlossmann,²⁵ J. Müller²⁶) Schlossmann explains this phenomenon as due to the higher fat content, with relation to the protein, which is found in mother's milk. A quantitative examination (Blauberg) of the feces resulting from these two diets shows that the acidity is due largely to volatile fatty acids. Hedenius²⁷ has shown that with the same diet (carbohydrate), he obtained an acid stool or an alkaline stool according to the age of the infant (two months or seven to ten months). The reaction of the feces of adults fed upon cow's milk is similar to that of the infant stool resulting from a similar diet, i. e., neutral to slightly alkaline (Rubner), although Lynch shows that the reaction may be slightly acid under such conditions.

A pathological condition of the intestinal tract may result in a change in the reaction of the feces. Those diseases associated with the poor utilization of the fats or in an increased fermentation give rise to an acid stool, while those diseases which result in an increased putrefaction are accompanied by stools having an alkaline reaction. Müller²⁸ and Schmidt and Strasburger report the reaction of fasting feces as slightly acid.

The exact hydrogen ion concentration of feces, so far as we have been able to find from an examination of the literature, has never been determined.

¹ Schmidt and Strasburger *Loc cit*, p. 107

²² Blauberg *Loc cit*

²³ Hellstrom *Archiv für Gynäkologie*, 1901 (cited by Hecht)

²⁴ Langstein *Loc cit*

²⁵ Schlossmann *Zentralbl für Kinderheilkunde*, 18, No. 7, 1906

²⁶ J. Müller *Loc cit*

²⁷ Hedenius *Archiv für Verdauungskrankheiten*, 111, p. 379, 1907

²⁸ Müller *Berliner Klin. Woch.*, 24, p. 433, 1887, *Archiv's Archiv*, cxxx1 (supplement), 1893

EXPERIMENTAL

Three men, C, V and E served as subjects in this investigation. Subjects C and V were used in the study of the effect of water drinking with meals. Subject C was twenty-nine years old and weighed 60 kg, while subject V was twenty-four years old and weighed 58 kg. Subject E, who was used in the fasting test, had been the subject of previous experiments in this laboratory.²⁹

The investigation upon subjects C and V was divided into five periods, the diet remaining uniform. The periods are given in Table I, p 135. Examinations were made of the stools of the last four periods.

The fasting experiment was divided as follows: a four-day preliminary period of high protein intake, a fasting period of seven days, a period of four days in which the subject ingested a low protein diet and a final period of four³⁰ days during which the diet was the same as that ingested during the preliminary period.

The diet was the same in character for each of the three subjects. The two men in the water drinking experiments ingested 400 cc milk, 100 grams of graham crackers, 15 grams of peanut butter and 25 grams of butter with each meal. The diet of subject E will be given in a forthcoming paper from this laboratory.³¹

The fecal extract, to be used in the determination of the hydrogen ion concentration was prepared as follows, 50 cc of $\frac{N}{2}$ Na_2SO_4 solution being used for the extraction, exactly 2 grams of moist feces³² was weighed out, by difference, into a mortar, and about 5 cc of the $\frac{N}{2}$ Na_2SO_4 solution added after which the feces were worked up with a pestle until the sample was in a fine homogeneous suspension. The remaining portion of the 50 cc of the

²⁹ Howe, Mattill and Hawk *Journ Amer Chem Soc*, XLIII, p 568, 1911, Mattill and Hawk *Ibid*, XLIII, p 1978, 1911. Unpublished experiments.

³⁰ In the case of the urine the period was five days in length (see Sherwin and Hawk, *unpublished*).

³¹ Sherwin and Hawk *Loc cit*.

³² The comparison was made upon the bases of 2-gram samples of moist feces. The variable moisture content of the feces seemed to preclude such a standard but careful consideration indicated that no other satisfactory basis was apparent.

Na_2SO_4 solution was then added and the whole thoroughly mixed together. This suspension was centrifugated and the supernatant liquid taken for the determination. The solutions prepared under these conditions were usually colored a light yellow to a dark brown and showed very little sediment upon standing.

The "true acidity" of the fecal extract is defined as the hydrogen ion concentration while the "titratable acidity" is the quantity of acid or alkali of known strength required to produce neutrality with respect to some indicator. Two methods of determining the true acidity are available, by the use of a series of indicators or by the aid of the hydrogen electrode. The indicator method³³ is very satisfactory when clear solutions can be obtained, however, when colored or turbid solutions are to be examined this method either fails or loses its accuracy. In the case of feces it is practically impossible, by filtration through paper or by centrifugation, to obtain an extract which is not colored and turbid.

The method of centrifugation offers the best means of preparing the fecal extract free from all of the heavier particles, and adapts itself especially to routine and clinical work. The determination of the hydrogen ion concentration by means of the hydrogen electrode offers the most accurate method of obtaining the true acidity of fecal extracts. In our work the determinations were all made upon fecal extracts prepared by centrifugation and the hydrogen ion concentration was determined with the hydrogen electrode.³⁴

The determination of the hydrogen ion concentration by means of the hydrogen electrode depends upon the difference of potential which exists between two hydrogen electrodes dipping into solutions of different concentrations. Knowing the difference of potential and the hydrogen ion concentration of one solution we can calculate the hydrogen ion concentration of the other solution according to the Nernst formula,

$$\tau = \frac{RT}{nF} \ln \frac{C_1}{C_2}$$

³³ Friedenthal *Zeitschr f Elektrochem*, v, p 113, 1904, Salm *Zeitschr f physikal Chem*, lvi, p 471, 1907, Michaelis and Rona *Zeitschr f Elektrochem*, xiv, p 251, 1908, Walpole *Biochem Journ*, v, p 207, 1910.

³⁴ We wish to thank Drs. E. W. Washburn and Grinnell Jones of the laboratories of physical chemistry and electro-chemistry for their courtesy in loaning us apparatus and in aiding us with many helpful suggestions.

This expression can be simplified in this case to $\pi = KT \log \frac{C_1}{C_2}$, where

$K = \frac{R}{F \times 0.4343}$ and is equal to 0.0001983, π is the difference of potential

between the two electrodes in solutions whose concentrations are C_1 and C_2 , R is expressed in joules per degree, T is the absolute temperature, n the valence is equal to unity and F is equal to 96,540 coulombs. The difference of potential was measured by means of the Poggendorf compensation method. A Lippmann electrometer was used to indicate the zero potential. The apparatus was sensitive to changes of 0.001 volt but readings were only recorded to 0.1 volt as this was sufficient for the purposes of this experiment.

The difference of potential between the two solutions was ordinarily small, consequently a Weston cell was introduced into the circuit of the concentration cells to increase the voltage of that circuit. In taking readings this cell was placed first in series with and then against the concentration cell, thus giving a check on the readings. The Weston cell was compared with a standard Weston cell both before and after a series of readings. The standard of comparison was a solution containing 0.2 mole Na_2HPO_4 and 0.1 mole NaH_2PO_4 .³⁵ This solution as has been shown by both Washburn and Henderson,³⁶ gives a hydrogen ion concentration of approximately 1×10^{-7} , i.e., it is neutral.

The feces were extracted with a $\frac{N}{2}$ solution of Na_2SO_4 . Such a concentration of Na_2SO_4 was selected in order that the concentration of the sodium ions should be approximately equal in both the standard and the unknown solutions, thus tending to reduce the solution-potential between them. The sodium sulphate solution served to neutralize the effect of the variations in electrolyte content of the feces and to carry the current and thus prevent changes in the concentration of the hydrogen ions around the electrodes. A saturated solution of sodium sulphate was used as the connecting solution between the feces extract and the standard phosphate solution.

The form of apparatus was that described by Salm.³⁷ This consisted of two U-tubes 18 mm in diameter, the arms of which were 60 and 80 mm in length respectively. The long arm of each U-tube was closed with a three-hole rubber stopper which held the platinum electrode, a tube for conducting the hydrogen to the electrode, one end of which was drawn out into a capillary and bent at an angle of nearly 180° and another glass tube bent at an angle of 90° which permitted the escape of the hydrogen from that arm of the U-tube. The two solutions were connected through the short arms of

³⁵ This solution has already been used in this laboratory for another purpose (see Hawk *Arch Int Med*, viii, p 552, 1911)

³⁶ Washburn *Journ Amer Chem Soc*, xxx, p 31, 1908, Henderson *Amer Journ of Physiol*, xxi, p 173, 1908

³⁷ Salm *Zeitschr f physikal Chem*, lvii, p 471, 1907

TABLE I

Hydrogen Ion Concentration of Feces Water Drinking (Hydrogen ion concentrations are expressed in moles per liter)

NUM BER OF STOOL	SUBJECT V			NUM BER OF STOOL	SUBJECT C		
	Weight of Feces	H ₂ O in Feces	H ⁺ ion Concentration		Weight of Feces	H ₂ O in Feces	H ⁺ ion Concentration
Moderate Water Drinking (ten days)							
	grams	per cent			grams	per cent	
3	101.5	79.6	7.1×10^{-8}				
4	153.5	82.4	1.7	1	103.5	76.2	1.9×10^{-8}
5	235.0	87.5	1.0	2	65.5	74.9	2.0
6	115.5	84.9	1.1	3	130.0	75.4	0.3
7	93.5	80.0	0.83	4	44.5	77.1	0.15
9	92.5	78.9	1.3	5	70.5	76.1	0.46
10	105.0	80.6	1.8	7	83.5	73.8	1.0
				8	50.0	73.4	0.98
				9-10	97.0	72.7	0.44
Average			1.3×10^{-8}	Average			0.90×10^{-8}
Normal Period (five days)							
1	48.5	79.0	0.65×10^{-8}	1	43.5	76.6	0.32×10^{-8}
2	56.5	75.8	0.40	2	43.5	73.3	0.41
3	244.5	80.7	0.60	3	60.5	69.6	0.28
4	154.5	82.4	0.62	4	73.5	70.3	0.33
5	115.5	80.8	2.4	5	136.0	72.3	0.17
Average			0.93×10^{-8}	Average			0.30×10^{-8}
Copious Water Drinking (five days)							
1	56.0	81.0	0.81×10^{-8}	1	57.5	76.2	0.96×10^{-8}
2	75.5	77.6	2.5	2	43.5	70.3	0.59
3	44.5	84.2	0.89	3	56.5	74.4	0.71
4	120.0	77.7	0.37	4	86.5	71.8	0.34
5	171.5	77.5	0.83	5	93.5	71.3	0.35
Average			1.06×10^{-8}	Average			0.59×10^{-8}
Normal Period (five days)							
1-2	162.5	79.7	0.78×10^{-8}	1	72.0	72.8	0.35×10^{-8}
3	124.5	80.8	0.71	2	66.5	72.8	0.20
4	56.5	78.9	1.0	3	72.5	74.8	0.38
5	214.0	83.9	1.0	4	74.5	76.8	0.37
				5	72.5	77.3	0.36
Average			0.89×10^{-8}	Average			0.33×10^{-8}

TABLE II

Hydrogen Ion Concentration of Feces Fasting (Hydrogen ion concentrations are expressed in moles per liter)

NUMBER OF STOOL	SUBJECT E		
	Weight of Feces	H O in Feces	H ⁺ ion Concentration
<i>Preliminary period (four days)</i>			
	<i>grams</i>	<i>per cent</i>	
2	166 0	77 4	5.0×10^{-8}
3	184 5	76 7	8 7
4	215 0	77 7	9 8
5	35 5	73 5	0 60
6	51 0	70 9	2 6
Average			5.3×10^{-8}
<i>Fasting (seven days)</i>			
1	81 0	88 7	1.4×10^{-8}
2	38 5	80 5	0 94
Average			1.1×10^{-8}
<i>Low Protein (four days)</i>			
1	44 0	81 7	0.63×10^{-8}
2	19 0	91 0	6 6
Average			3.6×10^{-8}
<i>Final Period (four days)</i>			
1	192 5	78 4	3.4×10^{-8}
2	230 0	85 2	2 1
3	86 5	80 1	0 53
4	135 0	77 4	0 87
5	180 5	77 2	1 6
Average			1.7×10^{-8}

the U-tubes by a glass tube of 10 mm diameter bent twice at right angles and which was filled with cotton saturated with a saturated solution of sodium sulphate

The hydrogen was generated from metallic aluminum and caustic potash. The stream of hydrogen was washed twice with a solution of sodium hydroxide and pyrogallol, then divided into two currents each of which was

passed through a set of absorption bulbs connected with one of the U-tubes of the Salm cell. The set of bulbs connected with the standard phosphate U-tube was filled with the phosphate solution and that before the fecal extract was filled with $\frac{N}{2}$ Na_2SO_4 solution. In this manner pronounced changes in the concentration of the solutions under examination were prevented. The two streams of hydrogen as they came from the concentration cells were united by a Y-tube attached to a bulb containing about 5 mm of mercury, thus insuring an equal pressure of hydrogen in each cell. The cells were kept in a room free from drafts and readings were taken at room temperature. The procedure after making the extraction was as follows: the phosphate solution was placed in one U-tube and an equal amount of feces extract in the other, the lower end of each tube was closed and hydrogen permitted to pass through the solution for at least three hours. At the end of this time the two cells were connected by means of the bent tube containing the saturated solution of Na_2SO_4 and the readings taken.

Blank tests were made upon the sodium sulphate solution and the phosphate solution. The results showed them to have practically the same potential, the readings varying between 0.0 and 0.003 volts in a series of five different tests. To prove that no change in the extract occurred upon standing at room temperature, solutions were permitted to stand for six hours after the readings were taken. The results upon these solutions showed no change in the hydrogen ion concentration.

DISCUSSION

The concentrations of hydrogen ion, are contained in Table I, p 135, and Table II, p 136, and are expressed in moles per liter.

A consideration of the data from the two water drinking experiments upon subjects C and V, Table I, p 135, indicates a hydrogen ion concentration or "true acidity" of between 0.3×10^{-8} and 1.0×10^{-8} , which represents a slightly alkaline solution.

In the case of subject V with the exception of the high value, 7.1×10^{-8} , the average hydrogen ion concentration for the period of moderate water drinking was 1.3×10^{-8} . Upon return to the normal diet this value dropped to an average hydrogen ion concentration of 0.93×10^{-8} which is a rather high value since for four days the hydrogen ion concentration averaged 0.57×10^{-8} and only upon the fifth or last day was there a significant change in the concentration. As the result of the ingestion of large amounts of water with meals the hydrogen ion concentration increased very slightly to an average of 1.06×10^{-8} . Neglecting the high value, however, for the second day of the period we obtain an average of 0.72×10^{-8} . Upon the return to the normal diet-

ary conditions we do not observe any distinct change in the hydrogen ion concentration, the final average being 0.89×10^{-8}

The hydrogen ion concentration of the feces obtained from subject C did not vary to any great extent during the course of any particular period. The greatest fluctuation occurred during the period of moderate water drinking, being between 0.15×10^{-8} and 2.0×10^{-8} moles of hydrogen ion per liter. The average for the period was 0.90×10^{-8} . Upon the return to the normal diet we find a lower value, the average being 0.30×10^{-8} . The hydrogen ion concentration increased under the influence of the ingestion of large quantities of water with meals to a value of 0.59×10^{-8} and subsequently decreased to 0.33×10^{-8} , when the normal diet was again resumed. This final value is a practical duplication of the value obtained in the other normal period.

The results obtained from subject V do not indicate conclusive changes in the hydrogen ion concentration of the feces due to the influence of water drinking since there are pronounced isolated variations in the hydrogen ion concentration. If these variations in the hydrogen ion concentration be omitted and we compare the average values for this experiment with those obtained from subject C we secure results which indicate that there was a slightly increased hydrogen ion concentration as the result of the ingestion of increased amounts of water with meals.

The increase was more pronounced during the early days of the period in each instance.

A comparison of the hydrogen ion concentrations of the feces of the normal period of subject E when taken into consideration in connection with the data obtained from the examination of the stools of the other subjects will be of interest inasmuch as they were ingesting similar diets. An examination of the data discloses the fact that the reaction varied with the individual. The water content did not seem to have any direct relation to the hydrogen ion concentration. This is shown very clearly from the fact that the stools of subject E yielded the maximum hydrogen ion concentrations whereas those of subject C yielded the minimum, notwithstanding the fact that the moisture values for the stools of the two subjects were very similar.

The uniformly slightly alkaline stools obtained in this experiment during the period of normal feeding were to have been expected

from the results of the findings of previous experimenters who have shown that an alkaline stool results from the ingestion of a milk (cow's) diet³⁸

Indicator papers were used by us to determine the acidity of the fecal extracts and while they give a rough estimate of the hydrogen ion concentration the results were not sufficiently accurate to show any distinction between the acidity of the individual stools although as the data obtained from the use of the hydrogen electrode show, there was a distinct difference between the stools of different men

The data from the fasting test give us information regarding the influence of pronounced variations in the dietary régime upon the reaction of the feces. Even when the subject passed in succession through periods of high protein feeding, of fasting and of subsequent low and high protein feeding, the reaction remained uniformly alkaline and with but comparatively small variations. The two fasting stools whose hydrogen ion concentrations were 1.4×10^{-8} and 0.94×10^{-8} which would be acid to phenolphthalein and alkaline to litmus, are to be considered as alkaline. This finding of an alkaline reaction in fasting feces is opposed to the finding of Müller³⁹ who states that the fasting feces from Cetti were acid in reaction. Schmidt and Strasburger⁴⁰ also report the acid reaction of fasting feces which they ascribe to the presence of fatty acids. No reference is given as to the source of their information.

SUMMARY

The hydrogen ion concentration of the feces of three men was determined, two in a series of water drinking experiments and the third in a fasting test, with the accompanying preliminary and final periods. The same type of diet was employed in the water experiments and in the preliminary and post-fasting periods of the fasting test. The hydrogen electrode (Salm type) was used to determine the actual hydrogen ion concentration and indicator papers were used to determine the approximate hydrogen ion concentration.

³⁸ (Blauberger, Hellstrom, Langstein, Schlossmann, J. Müller) *Loc cit*

³⁹ Müller *Loc cit*

⁴⁰ Schmidt and Strasburger *Loc cit*, p. 107

The reaction of the feces was uniformly alkaline, the hydrogen ion concentration varying between 0.15×10^{-8} and 9.8×10^{-8} . As the result of water drinking with meals there was a tendency for the hydrogen ion concentration to increase. Pronounced changes in the dietary régime, such as high protein, low protein and fasting did not affect the hydrogen ion concentration of the feces sufficiently to cause other than small variations in the uniformly alkaline reaction. As the result of fasting, the stools were alkaline in reaction (hydrogen ion concentrations of 1.4×10^{-8} and 0.94×10^{-8}) as opposed to the acid stools reported by previous investigators. The hydrogen ion concentration differs for the feces of different individuals living on the same diet.

CARBOHYDRATE ESTERS OF THE HIGHER FATTY ACIDS

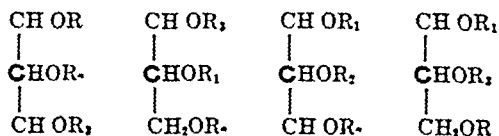
II MANNITE ESTERS OF STEARIC ACID

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Anyone who has given serious thought to the metabolism of the fats must have been impressed by the cumbrousness of the accepted theory of fat absorption as well as by the gaps in our knowledge of what happens to the fats after they enter the blood stream. Unless we assume some sort of protective mechanism, which allows only such fatty substances as admit both of easy emulsification and saponification to pass into the blood stream, it is hard to understand why it should be necessary for a fat to be broken down into its component parts to pass through one side of an intestinal cell, only to be immediately resynthesized on passage through the other side of the same cell. With the idea of getting additional evidence for or against the accepted theory of fat absorption, and to obtain some information with regard to fat transportation in the blood stream, a fatty compound was sought which had some characteristic physical or chemical property which would enable it to be traced through the processes of absorption and transportation. Attention was first turned to the possibilities of an optically active fat. Theoretically, as has been noted by several writers,¹ and as may be seen from the following formulas



¹ For discussion, see Lewkowitsch *Chemical Technology, etc of Oils Fats and Waxes*, 4th ed, 1, pp 8-13

an optically active fat is possible, but though many attempts have been made to prepare such a compound either from natural fats, or synthetically, so far none has been successful. Mixed esters, such as fulfil the conditions for optical activity are said to occur very frequently in natural fats, in fact Klimont² claims that most fats contain large amounts of mixed esters of this nature. Natural fats show optical activity occasionally, but this has been shown to be due, in all cases, either to accidental constituents, such as colors, or lipoid substances, or to the occurrence in the compounds of optically active acids, and never to molecular arrangement. Grun, whose name is most closely connected with the synthetic fats has prepared various types of glycerides,³ and has examined them for optical activity, but has not recorded any which show it. Our efforts in this direction were confined to the examination of cocoa-butter, which was said to consist largely of mixed esters,⁴ some of which should be optically active. Since the fat itself has no optical activity, if optically active glycerides are present they must be there in the racemic form. The attempt was made to resolve these into the active components by the use of various fat-splitting enzymes, by means of which Neuberg⁵ has been able to resolve some similar compounds. Although an occasional fatty residue was obtained which showed optical activity, boiling with bone-black in benzol caused it to disappear, thus showing that the activity was not due to the fat but probably to some coloring matter. The matter was dropped at this point for a time, and attention was next directed to the possibilities of a compound of a carbohydrate with a fatty acid. The interest in a compound of this sort is much increased by the relationship which has been repeatedly shown to exist between the carbohydrates and fats in metabolism, and which has been crystallized in the statement that "fats can burn only in the fire of the carbohydrates." Without going in detail into this question it is well known that in conditions where carbohydrate is withheld from the metabolism, as for instance in diabetes mellitus, starvation, etc., unburned residues of the fatty acid molecule— β -oxybutyric, acetoacetic acids and

² Klimont *Monatsh f Chem*, xxx, pp 341-46

³ Grun *Ber d deutsch chem Gesellsch*, xxxviii, p 2285

⁴ Klimont *Monatsh f Chem*, xliii, p 51

⁵ Neuberg and Rosenberg *Biochem Zeitschr*, vii, p 191

acetone,—appear in the urine, and that these may be caused to disappear (except in those cases where carbohydrate intolerance is extreme) by feeding a little carbohydrate

With these ideas in mind it was decided to attempt the synthesis and physiological study of some compounds of this nature. No method of synthesis being available which would guarantee the easy preparation of such large amounts of carbohydrate esters as would be required for a physiological study, it was decided to make a preliminary study using the hexatomic alcohol mannite, which is physiologically interchangeable with glucose,⁶ and which, because of its relatively stable nature lends itself readily to synthesis

An account of the method of synthesis and the preparation and description of one compound—mannid distearate—has been already reported in this journal,⁷ but a brief outline will not be out of place here. Mannite was dissolved in excess of concentrated sulphuric acid at 70°C, the stearic acid added and the mixture kept at 70° for three to four hours. The cooled mixture was extracted directly with ether and after washing the ethereal solution with water and freeing it from unused stearic acid by titration with alkali and filtering off the soap, the ether was removed by distillation. The compounds were purified by repeated fractionation with alcohol. By this method there was obtained along with the mannid distearate a considerable amount of ethyl stearate formed by interaction of the ether and stearic acid in presence of concentrated sulphuric acid and also varying amounts of another mannite ester which, being only slightly soluble in the ether, floated suspended in it.

The characteristics of the mannid distearate are briefly as follows. It is pure white, semi-translucent, brittle and amorphous, insoluble in water, slightly soluble in cold methyl and ethyl alcohols, readily soluble in them hot, soluble in cold ether, benzol and chloroform, heavier than water. Its melting point is 51° C. The optical activity in about 7 per cent solution in benzol is $[\alpha]_D^{25} = +64.9^\circ$. Its stearic acid content is 84.8 per cent (theoretical 83.8 per cent, and its molecular weight determined cryscopically in benzol

⁶ Külz *Pflüger's Archiv*, xxi

⁷ Bloor *This Journal*, vii, p. 427

was found to be 706 (theoretical 678) Ultimate analysis yielded the following figures

- (1) 0.1456 gram of ester yielded 0.1499 gram H_2O and 0.3940 gram CO_2
 (2) 0.1575 gram of ester yielded 0.1587 gram H_2O and 0.4270 gram CO_2

	Calculated for Mannid Distearate	Found	
		ⁱ	ⁱⁱ
C	74.04	73.80	73.97
H	11.50	11.43	11.20

Mannitan distearate

A sufficient quantity of the second mannite compound mentioned above, which was almost insoluble in cold ether, having been collected, its examination was undertaken. It was purified by many crystallizations from hot alcohol in which it is soluble and from which it separates on cooling in globules of microscopic needles. It is much less soluble in ordinary organic solvents than mannid distearate. It is very slightly soluble in cold alcohol, chloroform or benzol and while considerably more soluble in these solvents when hot it reaches only about 5 per cent in its best solvent—hot chloroform. Its melting point is $124^{\circ}C$ (uncorrected) when the crystals are used, but when cooled and remelted its melting point is $116.5^{\circ}C$.

Rotation It is weakly dextro-rotatory. The determination of its optical activity is a matter of considerable difficulty because of its slight solubility and low rotating power. Two grams of the substance were dissolved in 50 cc of chloroform at $50^{\circ}C$ and the reading was made in a water-jacketed, 1 dm tube at this temperature. The average reading for this solution was $+0.32^{\circ}$, from which $[\alpha]_D^{50} = +8.0^{\circ}$.

This figure is only approximate, first, because of the volatility of the solvent at this temperature, and second, because, although tried several times, an entirely clear solution was never obtained.

Mannitan distearate saponifies readily with alcoholic alkali and, after removal of the fatty acid, the solution on evaporation yields a syrupy liquid from which crystals of mannite separate on standing for a short time—much more readily than from the syrup obtained from mannid distearate.

Saponification On saponification and separation of the pure fatty acid as described for mannid distearate, the results were as follows

- (1) 1 1467 grams of ester yielded 0 9341 gram stearic acid = 81 45 per cent
 - (2) 1 1132 grams of ester yielded 0 9009 gram stearic acid = 80 91 per cent
 - (3) 1 0080 grams of ester yielded 0 8229 gram stearic acid = 81 64 per cent
- Calculated value for mannitan distearate = 81 62 per cent

Combustion 0 1700 gram ester gave 0 1692 gram H_2O and 0 4536 gram CO_2 .
0 1528 gram ester gave 0 1613 gram H_2O and 0 4049 gram CO_2 .

	Calculated for Mannitan Distearate	I	Found II	AVERAGE
C	72 41	72 76	72 26	72 51
H	11 49	11 06	11 72	11 44

Molecular Weight determination by elevation of boiling point of chloroform

(1) 0 4525 gram of substance in 29 43 grams chloroform gave an elevation of 0.080°

(2) 0 5517 gram of substance in 29 46 grams chloroform gave an elevation of 0.097°

	Calculated for Mannitan Distearate	I	Found II
Molecular Weight	696	704	706 6

The results of the analyses indicate that the substance is mannitan distearate and therefore closely related to mannid distearate,—apparently a hydrated derivative of it. The relationship appeared still more definite when it was discovered that by heating mannitan distearate to 200° for a short time a substance was obtained which had the same chemical composition as the mannid distearate, and in a general way the same properties. It was for a time considered to be identical with it, but a closer examination revealed marked differences and showed the substances were isomeric.

The Isomeric Mannid Distearate

Ten grams of pure mannitan distearate were heated at $200^\circ C$ in an air bath until the bubbles had ceased to come off and the colorless liquid had assumed the brown tint of incipient decomposition. On cooling, the liquid solidified to a light brown trans-

parent solid It was dissolved in benzol, treated with bone black, filtered, the benzol removed by evaporation and the substance purified by many precipitations from hot alcohol The fused solid is pure white, waxy and amorphous Melting point, 61.5°C (uncorrected) It is readily soluble in cold ether, benzol or chloroform, slightly soluble in cold alcohol, readily soluble in hot alcohol, from which latter it separates on cooling in non-crystalline spheres Like mannid distearate it is somewhat volatile with heat

Rotation It is strongly dextrorotatory Determinations made in benzol resulted as follows

(1) 0.8536 gram in 10 cc benzol in 1 dm tube, rotation = $+8.00^{\circ}$

(2) 0.5740 gram in 10 cc benzol in 1 dm tube, rotation = $+5.37^{\circ}$

$$[\alpha]_D^{20} = \frac{(1)+93.7}{(2)+93.55} \quad \text{Average} = +93.63^{\circ}$$

Combustion 0.1620 gram yielded 0.1687 gram H_2O and 0.4420 gram CO

	Calculated	Found
C	74.04	74.38
H	11.50	11.56

Molecular Weight determination by elevation of boiling point of chloroform 0.5242 gram in 29.66 grams chloroform gave an elevation of 0.098°

	Calculated	Found
Molecular Weight	678	666.7

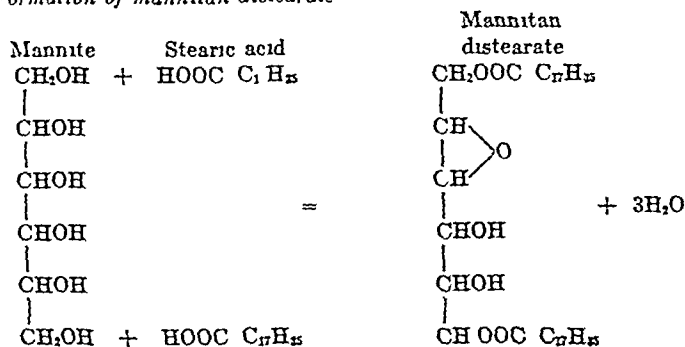
This substance is then probably an isomeric mannid distearate It differs from the mannid distearate first described in melting point, degree of optical activity and consistency It resembles it in solubilities and lack of crystallizing power The combined yield of esters by this method was about 50 per cent of the theoretical, calculating from the stearic acid

In later experiments to avoid the presence of ethyl stearate, which causes much trouble in the isolation of the mannid distearate, the esters were salted out by pouring the sulphuric acid digestion mixture into saturated ammonium sulphate Most of the sulphuric acid is thus removed and the salted-out mass, after filtering and washing several times with saturated ammonium sulphate and drying, could be extracted with ether or other organic solvents without danger of contamination with ethyl stearate When treated in this way, in the presence of excess of water only

mannitan distearate was obtained⁸ Yield—about the same as before

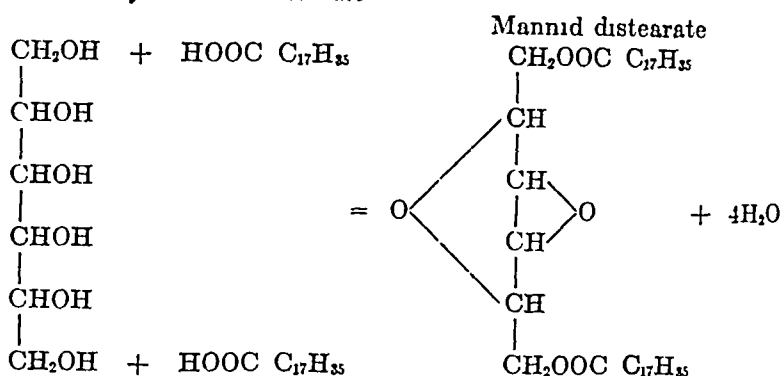
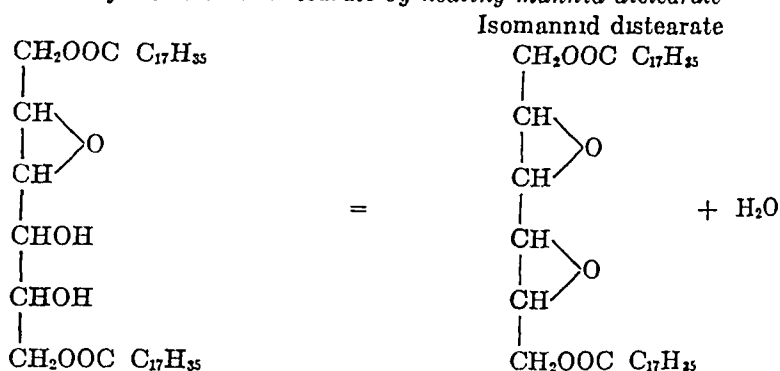
At the present time not enough is known of the chemistry of the mannite compounds to warrant the assignment of definite structural formulas to the stearates Glycerin when treated with the fatty acids in solution in concentrated sulphuric acid forms only the α - α -diacid ester, the acid uniting with the primary alcohol groups alone⁹ Since only the diacid esters of mannite are formed under the same conditions it is reasonable to assume that the combination takes place also at the primary alcoholic end groups In the sulphuric acid synthesis, mannite loses water from its hydroxyl groups with the formation of anhydrides—mannitan (loss of 1 molecule of water) and mannid (loss of two molecules of water) forms Nothing is known as to which of the alcohol groups takes part in the anhydrid formation The reactions may be represented as follows

Formation of mannitan distearate



⁸ It had been observed in the preparation of mannid distearate that the amounts of this ester and of the mannitan distearate varied considerably in different preparations, and the variation was reciprocal, i.e., when the amount of mannitan distearate was great, that of mannid distearate was small, and vice-versa The explanation of the variation and of the absence of mannid distearate in the above preparation seems to be that when the sulphuric acid mixture is poured into the salt solution, the excess of water present completely hydrolyses the mannid distearate to the mannitan form, while when the esters are extracted from the sulphuric acid mixture directly with ether, the change takes place to only a limited degree, owing to the small amount of water present

⁹ Grün *Loc cit*

Formation of mannid distearate*Formation of isomannid distearate by heating mannid distearate*

A closer study of the reaction was now undertaken with the object of improving the yield. The reaction mixture left after extraction of the esters was first examined. It was diluted with water and treated with powdered barium carbonate and hydrate until the sulphuric acid was removed, filtered, the precipitate of barium sulphate and carbonate washed with cold water, the filtrate and washings carefully neutralized and evaporated to small bulk. A considerable amount of a compound identified as the barium salt of ethyl sulphuric acid (derived from the ester) was recovered from the residues, but never any mannite compounds. This was remarkable, since according to the amount of esters obtained about 60 per cent of the mannite should have been present in the residues.

The reactions which take place when mannite is treated with concentrated sulphuric acid at different temperatures were next studied. This work has proved to be unexpectedly complicated

and, as it is still incomplete, a full report will be reserved for a later publication. The following facts are fairly well established. When mannite is dissolved in concentrated sulphuric acid at temperatures below 50°C , there is formed mannitan- (and possibly mannite-) disulphuric acid ester. At temperatures over 50°C , mannid-disulphuric acid ester is formed and, at the same time, there is a considerable condensation of the mannite molecules with the formation of a substance of the nature of a mannite ether, which has little or no power of forming esters. The formation of this substance at temperatures above 50° seemed to account for the low yield and accordingly a number of experimental syntheses were conducted at 38° to 40° , and the time increased to about twenty-four hours, the results of which experiments showed a yield of 85 per cent of the theoretical.

This modification was adopted in the preparation of most of the material for the digestion and feeding experiments.

The method of synthesis of mannitan distearate as finally carried out was as follows.

Ten grams of mannite was dissolved in 200 grams of concentrated sulphuric acid warmed to 40° , 30 grams of stearic acid stirred in, the flask stoppered and placed in an ordinary incubator at about $38^{\circ} \pm 2$. The mixture was shaken from time to time until the stearic acid was completely dissolved, and then left in the incubator over night. Next day it was poured in a fine stream with stirring into about a liter of cold saturated ammonium sulphate solution and after thorough stirring, set aside for the esters to separate out, after which it was filtered on a Buchner funnel and washed two or three times with saturated ammonium sulphate solution. The filtered mass was pressed as dry as possible, then treated with hot benzol on a water bath. After washing two or three times with hot water to remove any remaining sulphuric acid, the water was siphoned off and the benzol solution was allowed to cool, depositing the mannitan distearate, and retaining the excess of stearic acid in solution. After filtering from the benzol the ester was purified as before by precipitation from hot alcohol.

Digestion Experiments on Mannid and Mannitan Distearate with Lipases

The following lipase-containing materials were employed.

1. Pancreas powder from pigs' pancreas prepared according to Dietz¹⁰

¹⁰ Dietz *Zeitschr f physiol Chem*, 111, p 286

2 Glycerin extract from pigs' pancreas prepared according to Kanitz ¹¹

3 Water extract of pancreas—made by shaking the finely divided, fat-free pancreas with water, leaving over-night and using the turbid supernatant liquid

4 Human pancreatic juice ¹²

5 Castor bean powder prepared as follows

Large, fully ripe, castor beans were selected, the shells removed and the beans ground as fine as possible in a mortar. The thick paste was transferred to a wide mouthed bottle and extracted over night with a mixture of equal parts of alcohol and ether. Next morning the alcohol-ether mixture was shaken up and after standing a few seconds was poured off into another bottle, and with it the finer portion of the bean powder, which was now allowed to settle out. The coarse portions in the other bottle were drained, removed to a mortar, ground up again and returned to the bottle with the fine settlings from the alcohol-ether mixture. A second extraction was made, this time with ether alone. At the end of the extraction the mixture was well shaken and after standing a few seconds the ether was poured off, carrying with it in fine suspension, most of the bean powder. The remainder was ground up, and again shaken out with ether as before. Only the fine powder carried out in suspension in the ether was used for the work. It was filtered free from ether, dried and kept in a tightly stoppered bottle. The powder so prepared was very active and retained its activity undiminished for several months. This plant lipase differs from animal lipase in that it works best in a weakly acid medium, $\frac{N}{100}$. It also requires the presence of a small amount of acid for activation ¹³

The experiments were carried out in loosely stoppered test tubes holding about 50 cc, with the liquids saturated with chloroform, which according to Kikkoji ¹⁴ best prevents the action of bacteria with the least harm to the enzymes. Care was taken to obtain and preserve a good emulsion—the protein of the lipase preparation serving in most cases as the emulsifying agent. Parallel experiments were conducted, using cotton oil, both as a check on

¹¹ Kanitz *Zeitschr f physiol Chem*, lvi, p 483

¹² Obtained through the kindness of Dr Benj R Symonds from a case of pancreatic fistula in the general hospital at Salem, Mass. An account of the case is contained in the Thirty-fifth Annual Report of the Salem Hospital, 1909, p 29

¹³ Connstein, Hoyer and Wartenberg *Ber d deutsch chem Gesellsch* xlvv, p 3988

¹⁴ Kikkoji *Zeitschr f physiol Chem*, lxi, p 109

the activity of the enzyme and for comparison Blank experiments on the reagents alone were carried out with each experiment, under exactly the same conditions, and corrections made accordingly At the end of the time allowed for digestion the tubes were filled with absolute alcohol, shaken to loosen the digestion mixture and emptied into small beakers The tubes were rinsed out with two tubes full of alcohol and one of ether, the washings added to the liquid in the beakers and the whole titrated with normal alcoholic alkali, using phenolphthalein as indicator The end point chosen was the first rose color which lasted for one minute

The results of the experiments were as follows

Mannid Distearate (M P, 51° C)

With human pancreatic juice (1) Two grams mannid distearate melted with 5 cc hot water and shaken until emulsified, cooled (the emulsion remained), mixed with 5 cc of the human pancreatic juice, again shaken, and kept at 37° C over night

Titration 0.75 cc $\frac{N}{10}$ alkali correction for blank = 0.35 cc, weight of stearic acid set free = 0.11 gram, corresponding to 0.14 gram mannid distearate, digestion = 7 per cent

(2) One gram of ester was treated with water as in (1) and to it added 5 cc pancreatic juice and fifteen drops of ox bile, left over night at 37° C

Titration 0.90 cc $\frac{N}{10}$ alkali correction for blank = 0.35 cc, weight of stearic acid set free = 0.16 gram, corresponding to 0.2 gram ester, digestion = 20 per cent

(3) Two grams of mannid distearate, 5 cc pancreatic juice, 5 cc water, ten drops 5 per cent soap solution, left over night at 37° C

Titration 1.30 cc $\frac{N}{10}$ alkali correction for blank = 0.35 cc, weight of stearic acid set free = 0.28 gram, corresponding to 0.35 gram ester, digestion = 17.5 per cent

(A) *Experiment with cotton oil* to test the activity of the pancreatic juice Two grams cotton oil, 5 cc water, 5 cc pancreatic juice The whole well emulsified by shaking and left over night at 37° C

Titration 1.4 cc $\frac{N}{10}$ alkali correction for blank = 0.4 cc, weight of fatty acid as oleic acid = 1.128 grams, corresponding to 1.17 grams olein, digestion = 58 per cent

(4) A mixture was prepared by melting together 2.5 grams of cotton oil and 5 grams of mannid distearate, in the hope that a mixture of lower melting point would digest better Two grams of this mixture, emulsified with 5 cc of pancreatic juice and 5 cc of water, were kept at 37° C over night

Titration 3.35 cc $\frac{N}{10}$ alkali correction for blank = 0.35 cc Stearic acid = 0.85 gram

If 58 per cent of the cotton oil had digested as in (A) above there would be left about 0.41 gram as originating from the ester, corresponding to an ester value of 0.52 gram, a digestion of 28 per cent

(5) Two grams of the same mixture with 5 cc of water and 5 cc pancreatic juice, shaken to a permanent emulsion, and left overnight at 37° C

Titration 2.7 cc $\frac{N}{1}$ alkali corrected as in (3) shows a digestion of 0.35 gram of ester or 17.5 per cent

(6) One and four-tenths grams of mixture, 5 cc of water, 5 cc of pancreatic juice shaken to permanent emulsion and left overnight at 37° C

Titration 2.0 cc $\frac{N}{1}$ alkali digestion 0.252 gram of ester = 18 per cent

Experiments 1, 2 and 3 indicate that mannid distearate is slowly attacked by human pancreatic juice especially in the presence of bile

The results of Experiments 4, 5 and 6 are of doubtful value as evidence for digestion of the mannid distearate, for if the cotton oil were completely digested instead of the assumed 58 per cent, this showing would be eliminated

Castor bean lipase (1) One gram of mannid distearate, 0.5 gram of castor bean powder, 4 cc $\frac{N}{10}$ sulphuric acid, 5 cc water, shaken to a good emulsion (the protein of the bean is the emulsifying agent in this case) and let stand over night

Titration 1.3 cc $\frac{N}{1}$ alkali - 0.4 cc (blank) = 0.9 cc = 0.26 gram stearic acid = 0.35 gram of ester, digestion = 33 per cent

(A) Five cubic centimeters cotton oil, 0.5 gram castor bean powder, 4 cc $\frac{N}{10}$ sulphuric acid, 5 cc water, shaken to good emulsion, let stand over night at 37° C

Titration 9.0 cc - 0.4 cc (blank) = 8.6 cc, $\frac{N}{1}$ alkali = 2.44 grams oleic acid = 2.54 grams olein, digestion = 55.2 per cent

Mannid distearate digests fairly well with the castor bean lipase

Digestion Experiments with Mannitan Distearate (M P, 124° C)

Mannitan distearate when freshly prepared and moist will form an emulsion with water which will pass through a filter while hot, and is permanent on cooling. This material, spoken of as "ester suspension" throughout these experiments, was prepared from the crude salted out mass (see p 149) in this way. After draining on a filter and washing several times with water, it was stirred with excess of alcohol and let stand an hour or two and filtered. The washing with alcohol was repeated until the washings were no longer

colored. Washing with water was then resumed and continued until the washings were free from sulphates. The moist substance so obtained, free from fatty acid and salts, was stirred into boiling water until 100 cc of water contained 20 grams of the ester, calculated to dry weight. On cooling it was about the consistency of thick cream and if evaporation is prevented may be kept in this form for several weeks without any separation.

With castor bean powder (A) Test of activity of powder with cotton oil. Forty-eight hours at room temperature (18 to 20° C)

Four and six tenths grams (5 cc) cotton oil, 5 cc $\frac{N}{10}$ acid, 5 cc water shaken to good emulsion

Titration 9.71 cc - 1.5 cc (blank) = 8.21 cc $\frac{N}{10}$ alkali = 2.35 grams, oleic acid = 2.47 grams olein, digestion = 53 per cent

(1) Ten cubic centimeters of the ester suspension (containing 2 grams of dry ester) were mixed with 0.5 gram of the bean powder, 2 cc of $\frac{N}{10}$ sulphuric acid + 5 cc of water. Left at room temperature over night

Titration 1.5 cc $\frac{N}{10}$ alkali - blank = 1.5 cc of alkali, no digestion

(2) Ten cubic centimeters of the ester suspension together with 0.5 gram castor bean powder, 2 cc $\frac{N}{10}$ sulphuric acid, 4 cc water, at room temperature for twenty-eight hours

Titration 8.6 cc $\frac{N}{10}$ alkali - 3.3 cc (blank) = 5.3 cc $\frac{N}{10}$ alkali, corresponding to 0.14 gram stearic acid or 0.18 gram ester, digestion = 9 per cent

(3) Ten cubic centimeters of ester suspension, 0.5 gram castor bean powder, 2 cc $\frac{N}{10}$ sulphuric acid, 5 cc water, forty-eight hours at room temperature

Titration 11.2 cc - 9.91 cc (blank) = 1.29 cc $\frac{N}{10}$ alkali = 0.037 gram stearic acid = 0.047 gram ester, digestion = 2.5 per cent

These results indicate that mannitan distearate is not saponified by the castor bean lipase

With pancreas powder (A) Test of the powder with cotton oil. 0.5 gram pancreas powder, 5 cc water, 1 cc of 0.5 per cent Na_2CO_3 solution, 5 cc cotton oil, forty-eight hours at 37° to 38° C

Titration 105.6 cc - 31.9 cc (blank) = 73.7 $\frac{N}{10}$ alkali = 2.09 grams oleic acid = 2.174 grams oil, digestion = 46 per cent

(1) 0.5 gram powder, 5 cc water, 1 cc of 0.5 per cent Na_2CO_3 , 10 cc ester suspension (2 grams), forty-eight hours at 37 to 38° C

Titration 38.1 cc - 31.8 cc (blank) = 6.3 cc $\frac{N}{10}$ alkali = 0.193 gram stearic acid = 0.24 gram ester, digestion = 12 per cent

(2) The same amounts of material left six days

Titration 40.9 cc - 25.00 cc (blank) = 15.90 cc $\frac{N}{10}$ alkali = 0.45 gram stearic acid = 0.56 gram ester, digestion = 28 per cent

(3) The same mixture + 5 cc of bile left eight days at 37 to 38° C

Titration 77.74 cc - 30.45 cc (blank) = 47.29 cc $\frac{N}{10}$ alkali = 1.33 grams stearic acid = 1.69 grams ester, digestion = 84 per cent

Mannitan distearate is therefore slowly attacked by the enzyme contained in pancreas powder

Glycerin suspension of pancreas (A) Preliminary test of glycerin extract with cotton oil Ten cubic centimeters cotton oil neutralized with 4.5 cc $\frac{N}{10}$ alkali (previously determined as advised by Kanitz)¹⁵ 5 cc glycerin suspension of pancreas, forty-eight hours at 37 to 38° C

Titration 226.5 cc - 37.74 cc (blank) = 188.76 cc $\frac{N}{10}$ alkali = 5.36 grams oleic acid = 5.57 grams olein, digestion = 60 per cent

(1) Ten cubic centimeters ester suspension (2 grams), 2 cc of $\frac{N}{10}$ alkali, 5 cc glycerin extract Forty-eight hours at 37 to 38° C

Titration 52.56 cc - 35.58 cc (blank) = 16.98 cc $\frac{N}{10}$ alkali = 0.482 gram stearic acid = 0.61 gram ester, digestion = 30 per cent

(2) Ten cubic centimeter ester suspension, 5 cc glycerin extract, 5 cc ox bile, forty-eight hours at 37 to 38° C

Titration 56.15 cc - 39.16 cc (blank) = 16.99 $\frac{N}{10}$ alkali = 0.482 gram stearic acid = 0.61 gram ester, digestion = 30 per cent

The mannitan ester digests about one-half as well as cotton oil with glycerin extract of pancreas

Water extract of pancreas (A) Testing with cotton oil Five cubic centimeters water extract, 5 cc cotton oil, 5 cc $\frac{N}{10}$ Na_2CO_3 forty hours at 37 to 38° C

Titration 9.04 cc - 2.98 cc (blank) = 6.06 cc $\frac{N}{10}$ alkali = 1.71 grams oleic acid = 1.78 grams olein, digestion = 39 per cent

(1) Ten cubic centimeters water extract of pancreas, 10 cc ester suspension + 10 cc $\frac{N}{10}$ Na_2CO_3 five days at 37 to 38° C

Titration 3.81 cc - 2.98 cc (blank) = 0.83 cc $\frac{N}{10}$ alkali = 0.24 gram stearic acid = 0.30 gram ester, digestion = 15 per cent

¹⁵ Kanitz *Loc cit*

The results of the digestion experiments are summarized below

Mannid distearate

DIGESTIVE AGENT	NUMBER OF EXPERIMENT	PERCENT-AGE DIGESTION	TIME OF DIGESTION	REMARKS
Human pancreatic juice	1	7	Twenty-four hours	Cotton oil under similar conditions is digested to the extent of 58 per cent
	2	20	Twenty-four hours (with bile)	
	3	26	Twenty-four hours (with cotton oil)	
	4	17.8	Twenty-four hours (with cotton oil)	
	5	18	Twenty-four hours (with cotton oil)	
Castor bean lipase	1	33.2	Twenty-four hours	Cotton oil under similar conditions, 55.2 per cent digestion

Mannitan distearate

Castor bean powder	1	None	Twenty-four hours	Cotton oil under similar conditions 48 hours 53 per cent digestion
	2	9	Twenty-eight hours	
	3	2.5	Forty-eight hours	
Glycerin extract of pancreas	1	30	Forty-eight hours	Cotton oil under similar conditions, 48 hours, 60 per cent digestion
	2	30	Forty-eight hours (with bile)	
Water extract of pancreas	1	15	Five days	Cotton oil, under similar conditions, 2 days, 39 per cent digestion

CONCLUSIONS

1 Mannid distearate shows, with human pancreatic juice, a digestibility of about one-third that of cotton oil, with castor bean lipase about one-half that of cotton oil

2 Mannitan distearate does not seem to be attacked by the lipase of the castor bean, but with the various pancreas preparations, a digestibility of from one-fourth to one-half that of cotton oil was obtained

FEEDING EXPERIMENTS

Feeding experiments were made only with the mannitan distearate, since it alone could be easily prepared pure and in large quantity

No attempt was made to feed the crystallized ester alone, since it has been conclusively shown that even in the case of normal food fats, those of high melting point (as for instance tristearin) are utilized with great difficulty when fed by themselves, but when dissolved in the liquid fats they are well utilized by the animal organism ¹⁶

For this reason the mannitan ester was fed (1) as the "ester suspension" used for most of the preliminary digestion work with the pancreas derivatives, and which for these feeding experiments was made thicker so as not to make the food too liquid, and (2) dissolved in cotton seed oil. Mixtures with cotton oil containing different amounts of the ester and varying in consistency from soft lard to hard tallow were used. The crude ester was prepared as described on p 149 and the actual amount of ester in the sample fed was determined in each case by precipitation from hot alcohol and weighing the dried precipitate. The animals experimented on were cats which were prepared for the experiment by starving for two days before the feeding.

In most cases bone ash was given with the food, both to mark the feeding periods and to ensure well formed feces, but to make sure that all the undigested ester was recovered, the feces were collected from the time of feeding till one day after the bone ash had passed. The combined feces were ground in a mortar, then

¹⁶ Arnschink. *Zeitschr f Biol*, 1916, p 434

extracted three or four times with boiling alcohol, which removed all but traces of the undigested ester. The alcoholic extracts were allowed to stand over night, the precipitate collected on a weighed filter, washed with cold alcohol, dried and weighed. Mannitan distearate, as noted on p 144 is practically insoluble in cold alcohol, so that this simple procedure gives sufficiently accurate results.

EXPERIMENT 1 Young cat, weight 1 kilo, fed 40 grams of a mixture consisting of 22 grams hashed lean meat, 30 cc of the ester suspension (containing 2.44 grams of ester) and 6 grams bone ash.

Weight of ester fed = 1.70 grams, recovered from feces = 1.58 grams, retained 0.12 gram, or 7 per cent.

The ester suspension is not well utilized and the other experiments were made with the ester dissolved in cotton seed oil.

EXPERIMENT 2 The same cat fed the whole of a mixture consisting of 3.5 grams ester melted with 3 grams of cotton oil (yielding a tallow-like product), 12.7 grams of hashed lean meat, 3 grams bone ash and 1 cc of blood (to increase the palatability).

Weight of ester fed = 3.5 grams, recovered from feces = 2.04 grams, retained 1.46 grams, or 41.7 per cent.

EXPERIMENT 3 A full grown male cat, weight 3.5 kilos was fed 64.5 grams of a mixture consisting of 3.8 grams of ester dissolved in 10 cc of cotton oil (the solution when cold had the consistency of soft lard and melted readily in the fingers), 50 grams hashed lean meat, 10 grams bone ash, 3 grams blood—in all 76.8 grams.

Ester fed = 3.16 grams, recovered from feces = 2.00 grams retained 1.16 grams, or 36.7 per cent.

EXPERIMENT 4 Prolonged feeding of ester, using the same cat as in Experiment 3.

In this experiment the ester was fed to the cat every day for six days. Feces were collected daily at 10 a.m. and the amount of unused ester determined. To find out whether the bone ash had any effect on the amount of ester absorbed it was omitted on certain days. As may be seen from the results, the bone ash seemed to aid absorption, since the bone ash feces contained as a rule less ester than the others. Aside from the bone ash, the food was the same every day and consisted of 3.8 grams of ester in 5 grams cotton oil, 50 grams hashed lean meat, 4 cc of blood and on the days when it was fed 6 grams of bone ash.

First day—Fed as above with bone ash.

Second day—No bone ash.

Feces collected weighed 30 grams containing 1.9 grams of unabsorbed ester.

Ester retained

1.9 grams

Third day—6 grams bone ash

Feces collected weighed 22.4 grams containing 1.69 grams of unabsorbed ester

Ester retained 2.11 grams

Fourth day—No bone ash

Feces collected—14.5 grams, contained 2.27 grams of ester

Ester retained 1.53 grams

Fifth day—No bone ash

Feces collected 16.5 grams contained 1.90 grams of ester

Ester retained 1.9 grams

Sixth day—Bone ash in feed Last feeding day No defecation

Seventh day—Feces not collected until next day

Eighth day—Feces collected, weight 35.6 grams containing 3.9 grams esters

Ester retained from two days feeding, 3.7 grams

Ninth day—Feces collected, weight 13 grams

Contained no ester, therefore elimination is completed

To recover any ester which may not have been previously extracted, the extracted feces were combined and boiled out several times with small portions of alcohol. The extracts were combined cooled and the precipitates weighed. There was recovered in this way a total of 1.5 grams of ester from the week's feces.

The balance for the six days was as follows

Total ester fed = 6×3.8 grams	22.8 grams
Total ester recovered	13.16 grams
Total ester absorbed	9.64 grams
	= 42.3 per cent

EXPERIMENT 5 To determine what becomes of a large amount of ester fed at one time, the same cat as in Experiment 4 was fed a mixture consisting of 10.6 grams of ester dissolved in cotton oil together with 70 grams lean meat, 6 grams bone ash and 3 cc. of blood.

The feces were collected from the time of feeding until one day after the passage of the bone ash, the whole ground in a mortar and extracted several times with hot alcohol as usual

	Grams
Weight of ester fed	10.6
Ester recovered from feces	4.92
Retained	5.68
	or 53.6 per cent

Results of feeding experiments with mannitan distearate

	Per cent
(1) Ester suspension	absorbed 7
(2) Solution in cotton oil	absorbed 41.7
(3) Solution in cotton oil	absorbed 36.7
(4) Solution in cotton oil, week's experiment	absorbed 42.3
(5) Solution in cotton oil Large feeding	absorbed 53.6

The results of the feeding experiments confirm those of the digestion experiments with lipase mixtures, that mannitan distearate is somewhat digestible. The amount of digestion, even under the ideal conditions in the intestinal canal is, however, only about one-half that of an ordinary fat, a fact which may be due to several causes, among which are the high melting point of the ester used and possibly the occurrence of the ester in isomeric forms of unequal digestibility¹⁷. It seems desirable before any other physiological experiments are undertaken to try the digestibility of some esters of lower fatty acids, as for instance, of lauric and myristic, which should give products of lower melting point. Work along this line is now under way.

In conclusion, it is a pleasure to acknowledge indebtedness to Professor Folin, of the Harvard Medical School, for direction of the research, and for an ever ready sympathy and help throughout its many difficulties.

¹⁷ A suggestion made by Dr I. K. Phelps of Washington, D. C., during the discussion following a report of this work given before the Biological section of the American Chemical Society at Indianapolis, June, 1911.

PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS

SECOND PAPER

THE ORIGIN AND SIGNIFICANCE OF THE AMMONIA IN THE PORTAL BLOOD

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In our last paper¹ we reported experiments which showed that amino-acids are absorbed as such from the intestine and are transported unchanged to all the different tissues of the body. A number of additional experiments have since been made, all of which confirm our findings as to the transport of non-protein nitrogenous materials from the intestine to the tissues. The further discussion of this subject will be taken up in a subsequent paper. This paper, as indicated by the title, deals with the origin and significance of the ammonia in the blood, particularly the portal blood.

The present day concepts of immediate deamination of the food protein are in a large measure based on experiments by Nencki and Pawlow and their associates on dogs with an Eck fistula.² Their observation that such dogs are poisoned when given much meat, coupled with their discovery that the portal blood contains "colossal" quantities of ammonia as compared with the ammonia content of systemic blood, seemed to furnish a substantial experimental basis for their theory of ammonia intoxication, as well as for the view that there is a large normal transformation of ammonia into urea in the liver. The subsequent important discovery made by Kutscher that protein digestion is accompanied by an abun-

¹ This Journal, vi, p. 87, 1912

² Arch f exp Path u Pharm, xxvii, p. 161, 1896, and xxxvii, p. 26, 1896, Zeitschr f physiol Chem, xxv, p. 449, 1898, xxxv, p. 246, 1902

dant amino-acid formation fitted in perfectly with the findings of the St. Petersburg investigators. It suggested a more localized deamination, with the production in the intestinal wall of the ammonia which is found in the portal blood.

The subject is an important one, and was naturally one of the first problems which we endeavored to elucidate further by means of our more refined analytical methods.

There is no question about the fact that portal blood normally does contain more ammonia than the systemic blood. The actual figures for the ammonia in portal blood as well as in systemic blood have, however, become smaller and smaller with every improvement in the technique employed for its determination. The figures given in the experiments below show that the values which we now obtain are very much lower than any previously recorded.

It is well known that the pancreatic digestion is accompanied by the formation of small amounts of ammonia. Corresponding to this ammonia formation one would expect to find a certain amount of ammonia in the mesenteric vein of the small intestine. This we have found to be the case, but we have not found that the ammonia is materially greater there than in the portal vein, as it should be if the small intestine were the sole or the chief source of the ammonia in the latter, on the contrary it is sometimes even smaller in amount.

Turning from the mesenteric vein of the small to that of the large intestine we come upon an entirely different condition. The ammonia content of this blood is invariably greater than that found in the portal vein, and in all the animals so far examined we have found it greater than that obtained for the blood coming from the small intestine.

EXPERIMENT 1 Cat 33 (weight 2913 grams) had been fed with raw meat for two days, the last feeding being seventeen hours before the operation. At the end of the experiment the stomach was found empty, the duodenum almost empty, while the jejunum and ileum still contained some unabsorbed food. The large intestine was well filled with feces.

The following figures were obtained for the ammonia

	<i>Milligrams</i>
I Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the large intestine	0 44
II Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the small intestine	0 05
III Ammonia nitrogen per 100 cc of portal blood (drawn last)	trace only
IV Ammonia nitrogen per 100 grams of moist feces taken from the upper part of the large intestine	15 0

The ammonia in the portal blood was in this case reduced to almost nothing by shutting off the mesenteric blood. The other organs, the spleen, pancreas and stomach whose blood also enters the portal vein contained therefore practically no ammonia. This corresponds with the results obtained by direct determinations of the ammonia in the blood coming from these organs.

EXPERIMENT 2 Cat 34 (weight 1893 grams) This was a young animal which we had kept for twelve days on a diet of dextrose and cream in order to reduce the store of non-protein nitrogenous materials in the blood tissues.

The results for the ammonia in the blood are as follows

	<i>Milligrams</i>
I Ammonia nitrogen per 100 cc of portal blood (drawn first)	0 13
II Ammonia nitrogen per 100 cc of mesenteric blood from the large intestine	0 24
III Ammonia nitrogen per 100 cc of mesenteric blood from the small intestine	0 18
IV Ammonia nitrogen per 100 cc of portal blood (drawn last)	0 07
V Ammonia nitrogen per 100 grams of feces taken from the ascending colon	4 7

EXPERIMENT 3 Cat 35 (very large) This animal was obtained from Dr Cannon in an anaesthetized condition (urethane). He had evidently been fed very heavily (the day before) for his stomach and small intestine were filled with meat and the large intestine was gorged with fecal matter. The odor from this cat was unusually foul.

The analytical results are as follows

	<i>Milligrams</i>
I Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the large intestine	1 6
II Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the small intestine	0 77
III Ammonia nitrogen per 100 cc of portal blood (drawn last)	0 1

EXPERIMENT 4 Cat 36 (weight 3093 grams) when opened was found to have the stomach empty, a moderate amount of food in the small intestine and a small amount of feces in the large intestine

The analyses of the blood gave the following results

	<i>Milligrams</i>
I Ammonia nitrogen per 100 cc of portal blood	0 22
II Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the large intestine	0 44
III Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the small intestine	0 41
IV Ammonia nitrogen per 100 cc of blood from the splenic vein	0 05
V Ammonia nitrogen per 100 cc of blood from the pancreatic duodenal vein	0 26
VI Ammonia nitrogen per 100 cc of carotid blood	0 03

EXPERIMENT 5 Cat 37 (weight 1623 grams) had been abundantly fed with meat for four days, the last time twenty-four hours before the experiment The stomach and the small intestine were empty and dry, the large intestine was well packed with feces

The blood analyses are recorded below in the same order in which the samples of blood were drawn

	<i>Milligrams</i>
I The ammonia determination in the portal blood miscarried	
II Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the large intestine	0 58
III Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the small intestine	0 31
IV Ammonia nitrogen per 100 cc of carotid blood	0 07
V Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the large intestine (thirty minutes later than II)	0 97
VI Ammonia nitrogen per 100 cc of blood from the splenic vein	trace only
Ammonia nitrogen per 100 grams of moist feces from the ascending colon	19 0
Ammonia nitrogen per 100 grams of moist feces taken near the rectum	19 6

The interesting point to be noted in this experiment is the unmistakable rise in the ammonia content of the blood which remained stagnant in the mesenteric vein of the large intestine during the time (about thirty minutes) which elapsed between the

first and the second withdrawal of blood from this vein. One of the assumptions frequently advanced in explanation of failures to find in the blood the products which are absorbed from the intestine is the velocity of the blood stream. That assumption carries with it another, namely, that the products absorbed are immediately and completely removed from the blood by some other tissue or organ, so as to prevent their accumulation. Whether the kidney activity is or is not eliminated, this condition is not fulfilled either for glycocoll or for urea and for ammonia it holds only within certain narrow limits. These limits are, however, easily exceeded as shown by the figures for the ammonia obtained in the following experiment.

EXPERIMENT 6 Cat 14 (weight 1970 grams) received in the ligatured small intestine 105 cc of a pancreatic self-digestion mixture containing about 0.8 gram of nitrogen, one-fifth of which consisted of ammonia.

	<i>Milligrams</i>
I Fifteen minutes after the injection the ammonia nitrogen per 100 cc of portal blood was	4.0
II The ammonia nitrogen per 100 cc of carotid blood taken about the same time was	0.4

In our first paper we showed that glycocoll is absorbed as such, and we further drew the conclusion that it is not appreciably deaminized while passing through the liver. The following experiment shows that glycocoll is not appreciably deaminized while passing through walls of the small intestine.

EXPERIMENT 7 Cat 38 (weight 2896 grams) had been well fed on meat for some time before being taken for the experiment. Five grams of glycocoll, dissolved in 50 cc of warm water, were injected into the ligatured small intestine (for anaesthetic we used ether and morphine). Half an hour later we began to take blood and obtained the following analytical results.

	<i>Milligrams</i>
I Ammonia nitrogen per 100 cc of portal blood	0.32
II Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the large intestine	0.53
III Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the small intestine	0.28
IV Ammonia nitrogen per 100 cc of carotid blood	0.03
V Glycocoll nitrogen absorbed	238.0

Since asparagine has an amide group as well as an amino group, the absorption of asparagine ought to be accompanied by an unmistakable increase in the ammonia of the portal blood, and by an excessive amount of ammonia in the mesenteric vein of the small intestine if any special deamination process is localized in the cell wall of the small intestine. The following experiment indicates clearly that to split off NH_2 groups hydrolytically is not one of the special functions of that tissue. There is no increase in the ammonia of the portal blood and the ammonia in the mesenteric vein is no larger than that of the portal blood.

EXPERIMENT 8 Cat 39 (weight 1043 grams), a very young animal, had been well fed for three days but during the last twenty-four hours had received no food. After etherizing and taking a sample of normal portal blood (by way of the splenic vein and without interrupting the circulation even in the latter) we injected 5 grams of Kahlbaum's asparagine dissolved in 50 cc of warm water. In order to allow the asparagine absorption to reach a high level we waited twenty minutes before beginning to collect blood. The following analytical results were obtained.

	<i>Milligrams</i>
Asparagine nitrogen injected	1077 0
Total nitrogen recovered	620 0
Asparagine nitrogen absorbed	457 0
I Ammonia nitrogen per 100 cc of portal blood before the injection	0 34
II Ammonia nitrogen per 100 cc of portal blood twenty minutes after the injection	0 36
III Ammonia determination in large intestine lost	
IV Ammonia nitrogen per 100 cc of blood from the mesenteric vein of the small intestine twenty-four minutes after the injection	0 38
V Ammonia nitrogen per 100 cc of carotid blood	0 08
VI Ammonia nitrogen per 100 grams of feces taken from the ascending colon	23 0

The large intestine clearly is the chief or at least the most constant source of the ammonia found in the portal blood. The reason for this is practically self-evident. The large intestine is the chief seat of bacterial action and as many of the bacteria, such as the *B. coli*, rapidly produce ammonia from albuminous materials, especially in the absence of carbohydrates, the condition in the

large intestine is ideal for the production of ammonia. Further, since the large intestine is practically never empty, there are always present the conditions for this ammonia formation, and that is why the ammonia in fasting animals is often as abundant in the portal blood as during digestion.

The total amount of ammonia which reaches the portal blood is, it will be noted, not very large, and it is extremely unlikely that this ammonia is the cause of the disturbance, produced by meat feeding in dogs with an Eck fistula. On the other hand, since this ammonia is not elaborated in the walls of the intestine as a part of the normal animal metabolism, but clearly comes straight from the fecal matter in the large intestine, it is not at all strange that dogs with Eck fistulas do not thrive on much meat. No one would suppose that the ammonia is the only product absorbed from that region. The Eck fistula dogs seem to furnish the first really definite illustration of "auto-intoxication" by way of the large intestine. The definitely fecal breath met with in many persons with "indigestion" acquires a somewhat unpleasantly definite significance in this connection.

Whether this and other symptoms of indigestion are due to the excessive production of putrefactive decomposition products in the large intestine or to an unusual failure of the liver to render those products harmless is an open question. But it looks at all events as if one of the most important functions of the liver is to dispose of the toxic materials coming from the large intestine.

As an essential part of animal metabolism the portal ammonia is hereby largely robbed of the peculiar interest which has been attached to it for the past fifteen years, and since the amount of ammonia in other blood is almost infinitesimal under ordinary normal conditions this too becomes a rather unimportant feature of normal metabolism. The ammonia in the tissues, the ammonia of experimental acidosis and certain obvious clinical applications remain to be investigated. We have already begun on this work, but some little time will necessarily elapse before we can report upon it.

FASTING STUDIES VII

THE PUTREFACTION PROCESSES IN THE INTESTINE OF A MAN DURING FASTING AND DURING SUBSEQUENT PERIODS OF LOW AND HIGH PROTEIN INGESTION

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INTRODUCTION

The course of the processes of intestinal putrefaction in the fasting organism has been but very little studied. One of the earliest investigations of prime importance was made by Friedrich Müller in connection with one of Cetti's fasts¹. Of course at the time these tests were made the output of total ethereal sulphate was considered to be the index of the extent of putrefaction within the intestine. However, in the investigation under consideration Müller determined the output of urinary indican as well as the total ethereal sulphate output. We may therefore follow the course of intestinal putrefaction. It is a surprising fact that Cetti's urines were free from indican after the third day of fasting. Upon the ingestion of food subsequent to the fast the indican was again present. Inasmuch as juices and secretions containing protein material are poured into the intestine even during periods of inanition it is surprising that no traces of indican were detected in the urine subsequent to the third fasting day. In this particular fast the last stool from the feeding period was not passed until the seventh day of the fast, a fact which would naturally tend toward the absorption of increased quantities of indol and the consequent augmentation of the indican output.

¹ Müller *Berl Klin Wochenschr*, xxiv, p 433, 1887, *Virchow's Archiv*, p 131, supplement, 1893

According to Weber² the excretion of total ethereal sulphate continues during fasting but is notably decreased as has been shown in experiments on both Cetti and Succì

Baumstark and Mohr³ have offered evidence from fasting studies in favor of the theory that indican may be considered an index of intestinal putrefaction. They determined that putrefactive processes continue in the intestine of fasting animals so long as the fasting feces are retained in the intestine. After the excretion of such feces, however, the urine contained no indican. These investigators argue that their observations furnish verification for the general belief that indol formed in the intestine is the sole source of the indican content of the urine and that it does not arise from the cleavage of tissue protein during fasting (Folin⁴ and others)

Although the relationship between fecal indol and urinary indican is almost universally accepted there is occasionally reported a finding which calls this relationship into question. Such a finding has been reported by v Moraczewski,⁵ who failed to show any relationship between the urinary indican and the fecal indol. The Ehrlich reaction was found to go parallel with the indol content of the feces and is consequently considered a measure of putrefaction. He further found that indol was increased when a carbohydrate diet was fed—a finding widely at variance with our present theories.⁶ Bickel⁷ has recently suggested the volatile fatty acid content of the feces as a measure of intestinal putrefaction.

DESCRIPTION

The object of the investigation was to study the influence of fasting and the subsequent feeding of low and high protein diets upon the course of intestinal putrefaction. The subject was a man (E) weighing 76.6 kgs at the opening of the fast. During a preliminary control period of four days he was maintained upon the following uniform diet: 600 grams graham crackers, 1350

² Weber *Ergeb d Physiol*, p 718, 1902

³ Baumstark and Mohr *Zeitschr f exp Path u Ther*, III, p 687, 1906

⁴ Folin *Amer Journ of Physiol*, III, p 99, 1905

⁵ v Moraczewski *Arch f Verdauungskranh*, XIV, p 375, 1908

⁶ Kendall *Journ Med Research*, XIV, p 411, 1911

⁷ Bickel *Arch malad l'app digestif*, v, No 11, p 589, 1911

grams whole milk, 75 grams butter, 150 grams peanut butter, 1050 cc water (300 cc at meal time and 750 cc between meals) This diet contained 21.86 grams of nitrogen (136 grams protein) and possessed an energy value approximating 6000 calories. The subject was therefore ingesting 1.77 grams of protein and nearly 80 calories per kilogram of body weight. The fast was seven days in length, the daily water ingestion being 1500 cc. The subject had undergone a fast of similar length⁸ during the previous year. On that occasion however he passed into the fast from a protein plane less than two-thirds as high (14.04 grams nitrogen) as that utilized in the present instance, i.e., 21.86 grams. The present fasting interval of seven days was followed by a low protein interval of four days in which 5.23 grams of nitrogen was ingested per day and this low protein plane was in turn followed by a five-day period during which the original high protein diet (21.86 grams nitrogen) of the pre-fasting interval was ingested.

The urine was collected in twenty-four hour samples. The indican content of the urine was taken as the index of intestinal putrefaction.⁹ Determinations of indican were made in duplicate upon each urine sample by the method of Ellinger.¹⁰ This method and others which have been suggested for the quantitative determination of indican has been discussed in a previous article from this laboratory.¹¹ In the present instance each cubic centimeter of the diluted Wang solution¹² employed in the titration was found upon standardization to be equivalent to 0.221 mgs of indigo or 0.424 mgs of indican. The total ethereal sulphate output was also determined for each day of the experiment, the method employed being that of Folin.¹³

DISCUSSION

The experimental data obtained from the analysis of the urines collected during the four periods of the experiment are given in the table on p. 172. The indican output is there expressed in milli-

⁸ Howe, Mattill and Hawk. *Journ Amer Chem Soc*, **xxviii**, p. 563, 1911.

⁹ Folin. *Amer Journ of Physiol*, **xiii**, p. 99, 1905.

¹⁰ Ellinger. *Zeitschr f physiol Chem*, **xxviii**, p. 192, 1903.

¹¹ Hattrem and Hawk. *Arch of Int Med*, **xii**, p. 610, 1911.

¹² Wang. *Zeitschr f physiol Chem*, **xxv**, p. 409, 1898.

¹³ Folin. *Amer of Physiol*, **xiii**, p. 52, 1905.

Indican and Ethereal Sulphates

Subject E

DAY OF EXPERIMENT	VOLUME OF URINE	TOTAL ETHEREAL SO_2	INDICAN	TOTAL ETHEREAL SO_2 INDICAN SO_2
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Preliminary Period—Four Days

	cc	mgs	mgs	
1	1200	158 2	42 2	11 78 1
2	1350	155 4	34 1	14 32 1
3	1210	164 8	61 6	8 41 1
4	1150	138 2	57 7	7 53 1
Average	1228	154 2	48 9	9 88 1

Fasting Period—Seven days

1	990	134 9	49 0	8 66 1
2	1345	114 5	60 5	5 95 1
3	1540	107 9	42 6	7 95 1
4	1340	92 3	26 3	11 04 1
5	1410	65 2	23 1	8 84 1
6	1015	18 6	15 4	3 79 1
7	1120	10 6	13 7	2 42 1
Average	1251	77 7	32 9	7 41 1

Low Protein Final—Four days

1	900	70 1	27 7	7 95 1
2	1000	63 9	64 9	3 10 1
3	1240	73 0	114 1	2 01 1
4	1330	117 6	89 6	4 12 1
Average	1118	81 2	74 1	3 44 1

High Protein Final—Five days

1	970	123 5	65 2	5 96 1
2	1420	132 0	84 4	4 92 1
3	2530	135 0	103 6	4 10 1
4	1550	197 3	69 3	8 94 1
5	2175	216 6	75 3	9 04 1
Average	1729	160 9	79 6	6 34 1

grams per day, the daily total ethereal sulphate excretion is expressed in milligrams of SO_3 and the relationship between total- SO_3 and indican- SO_3 is represented by means of the ratio "Total-ethereal- SO_3 : Indican- SO_3 ."

Indican The average daily output of indican for the preliminary period was 48.9 mg. This value is considerably lower than the values previously obtained for subject E in connection with the preliminary periods of two previous experiments¹⁴. The indican values in the instances mentioned were 69.2 mg and 67.3 mg and were obtained preliminary to some water drinking tests. The fasting study here reported was made several months later and in the interim the subject consumed rather large quantities of water daily. For this reason the lowered indican value obtained previous to the fast may possess considerable significance. It certainly indicates that there was less intestinal putrefaction and probably more efficient absorption. These facts carry added force when it is found that the low indican values were obtained during the feeding of a diet which contained approximately one-third more protein per day than in the previous instances cited. The actual nitrogen values of the diets were 21.86 grams and 14.76 grams¹⁵. There was no retention of feces in either experiment. All the associated facts mentioned above form a rather important verification of a conclusion reached by Hattrem and Hawk in connection with certain studies on water drinking, *i.e.*, "the drinking of copious or moderate volumes of water with meals decreases intestinal putrefaction as measured by the urinary indican output"¹⁶.

During the fast there was a progressive decrease in the excretion of urinary indican from the second day to the end of the fasting interval. The output for the second day was 60.5 mg whereas that for the last day was 13.7 mg, the values for the intervening days being intermediate in character. This pronounced decrease in the amount of indican excreted during the fast was of course the natural outcome of the non-ingestion of food. Inasmuch as the putrefaction processes and the consequent formation of indol in the lumen of the intestine are dependent entirely upon the pas-

¹⁴ Hattrem and Hawk. *Loc cit*

¹⁵ Mattill and Hawk. *Journ Amer Chem Soc*, **xxiii**, p 1999, 1911

¹⁶ Hattrem and Hawk. *Loc cit*

sage of protein material into the intestine it was to be expected that the withdrawal of food from the subject would result in a marked lowering of the indican values of the fasting urines. Various juices and secretions however, continue to be poured into the intestine during fasting and the protein constituents of the unabsorbed portion¹⁷ of these fluids would form a medium for the development and activity of the varied types of intestinal bacteria, among them the indol-formers¹⁸. For this reason even after prolonged fasting it is probable that there is ordinarily not a complete cessation of intestinal putrefaction. This would be particularly true in case the fasting subjects drank rather large volumes of water each day of the fasting interval as did subject E. It has been demonstrated quite conclusively that water ingested in large amount causes an increased outpouring of both the gastric¹⁹ and the pancreatic secretions²⁰.

It is evident from a consideration of the tabulated data that intestinal putrefaction was more pronounced in the post-fasting period of low protein ingestion than in the pre-fasting interval of high protein ingestion. What has caused this pronounced alteration in the relation existing between the diet and the accompanying intestinal putrefaction? That retention of the feces and the consequent more abundant formation of indol cannot be given any consideration in this connection is apparent from the fact that defecations occurred with signal uniformity from day to day. It might appear therefore at first thought that absorption of the protein constituents of the diet had been less efficient after the fast, thus giving the putrefactive bacteria a more copious quantity of digestion products to utilize in the formation of indol. If this factor is to assist in the explanation of the high plane of the indican excretion which is in evidence immediately following the fast it is apparent that there must have been a most unusual and pronounced derangement of the absorptive mechanism. This conclusion must follow from the fact that intestinal putrefaction was 50 per cent

¹⁷ Mosenthal *Proc Soc Exp Biol Med*, viii, p 40, 1910

¹⁸ Herter *Bacterial Infections of the Digestive Tract*, p 263

¹⁹ Pavlov and Khizhin. From Pavlov's *The Work of the Digestive Glands*, 2d Ed., p 112, 1910, Foster and Lambert *Journ of Exp Med*, v, p 820 1910, Wills and Hawk *Proc Amer Soc Biol Chem*, ii, p 29, 1911

²⁰ Pavlov *Loc cit*, p 144, Hawk *Arch of Int Med*, viii, p 382, 1911

greater when but 5.23 grams of nitrogen was passed into the gastrointestinal tract than it was when 21.86 grams of nitrogen was ingested

If defective absorption is to explain the variation in the indican values just discussed, it would be logical to expect a proportionate increase in the total nitrogen content of the feces. The data for fecal nitrogen²¹ show that there was no such increase. Data obtained from experimentation upon subject E in this and other connections seem to indicate that there is of necessity no uniform relationship between the urinary indican excretion and the output of bacteria in the feces, even when the diet of the subject is of the same general character in each instance.²² For example in the preliminary period of one of the experiments previously reported on subject E the average weight of dry bacteria excreted per day was about 9 grams with an accompanying indican value of 67.3 mgs, whereas in the present instance the bacterial value was approximately 14 grams and the indican value 48.9 mg. In further attempting to explain the comparatively low indican values for the high protein preliminary period it may be suggested that such indol as was formed was perhaps not efficiently absorbed, thus limiting the resultant indican values. The validity of this latter contention could easily have been determined, at the time, by examining the individual stools for indol. Unfortunately, however, this was not done inasmuch as the direct bearing of such observations was not foreseen until the data cited had been obtained and it was then too late to hope to secure any accurate indol data from the stools in question.

It is possible that the indol-forming organisms may have been more resistant to the rigors of the fasting régime and therefore as the fast progressed they formed, day by day, a progressively increasing proportion of the intestinal flora. Finally at the end of the fast, although the actual mass of the flora had become greatly decreased from the level of the preliminary period, the major part of the flora was now composed of efficient indol-forming organisms. Upon the entrance of the products of the digestion of protein food into the intestine, although small in quantity (low protein diet) it was nevertheless accompanied by the very rapid and

²¹ Blatherwick and Hawk. Unpublished

²² Kendall *Journ of Med Research*, xxi, p. 411, 1911

complete absorption and detoxication of the resultant indol. The above series of associated factors may have had an important bearing upon the ultimate production of the relations existent between the urinary indican and the fecal bacteria.

Ethereal Sulphate There was during the fast a close parallelism between the course of the indican excretion and that for total ethereal sulphate. With the opening of the subsequent feeding period, however, the uniform relation ceased. For example, upon the first day of the low protein feeding period, the output of ethereal sulphate was increased nearly *seven-fold* above the quantity excreted on the last day of the fast whereas the indican value was only *doubled* in the same interval. Furthermore as the subject passed from the low protein period to the high protein period his average daily ethereal sulphate value was increased about 100 per cent whereas his average daily indican value was but slightly augmented. Recent demonstrations of this lack of relationship have been submitted by Salant and Hinkel²³ and by Hattrem and Hawk²⁴.

When the data for the ratio between total ethereal-SO₃ and indican-SO₃ are examined it is noted that there was a marked drop in the ratio during the closing days of the fast. On the seventh day of fasting approximately 40 per cent of the total quantity of ethereal SO₃ excreted in the urine was in the form of indican-SO₃ whereas only about 10 per cent was excreted in this form in the urine of the fourth fasting day. It is apparent therefore that notwithstanding the fact that there was a close parallelism in the course of the excretion of indican and the other ethereal sulphates during the fast nevertheless there was a less pronounced inhibition of the activity of the factors intimately associated with the production and excretion of indican than of those factors regulating the output of the other ethereal sulphates.

SUMMARY

The subject of the experiment was a man weighing 76 kilos.

Intestinal putrefaction as measured by the output of urinary indican was markedly decreased during the fasting interval. The

²³ Salant and Hinkel *Journ of Pharm and Exp Ther*, 1, p 493, 1910

²⁴ Hattrem and Hawk *Loc cit*

seventh fasting day showed an indican excretion amounting to 13.7 mg as against an output of 60.5 mg for the second fasting day. During the post-fasting interval of low protein ingestion putrefaction was increased in a very pronounced manner, the indican values rising far above those obtained during the normal period preceding the fast. The average daily indican output was but slightly higher during the period of high protein ingestion than during the low protein period.

The indican data for the preliminary period when taken into consideration in connection with other similar data collected previous to certain tests upon the influence of a high water ingestion furnish an important verification of a conclusion previously reported from this laboratory to the effect that "The drinking of copious or moderate volumes of water with meals *decreases* intestinal putrefaction as measured by the urinary indican output."

It was demonstrated that intestinal putrefaction was 50 per cent greater when but 5.23 grams of nitrogen was passed into the gastro-intestinal tract after the fast than it was when 21.86 grams of nitrogen was ingested before the fast.

Data from this and previous experiments along similar lines made upon subject E seem to indicate that there is of necessity no uniform relationship between the urinary indican excretion and the output of bacteria in the feces, even when the diet of the subject is of the same general character.

The indican value for the high protein period subsequent to the fast was approximately 60 per cent higher than the indican value for the preliminary period notwithstanding the fact that the ingested diet was identical in kind and quantity in the two instances.

On the seventh day of fasting approximately 40 per cent of the total quantity of ethereal- SO_3 excreted in the urine was in the form of indican- SO_3 , whereas only about 10 per cent was excreted in this form in the urine of the fourth fasting day.

ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS

VI THE PROTEINS OF OX-SERUM, A NEW OPTICAL METHOD OF DETERMINING THE CONCENTRATIONS OF THE VARIOUS PROTEINS CONTAINED IN BLOOD-SERA

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1 INTRODUCTION

The influence which is exerted by the various proteins of blood-serum upon the refractive indices of their solutions was first systematically investigated by Reiss¹. This observer fractionated the proteins of horse and human sera in the following way. The globulins were precipitated by ammonium sulphate, "Euglobulin" being precipitated by 32 to 36 per cent saturation of the serum with ammonium sulphate, "Pseudoglobulin I" by 36 to 39 per cent saturation, and "Pseudoglobulin II" by 42 to 50 per cent saturation. Each of these fractions was dissolved in distilled water and reprecipitated twice, the final solutions thus obtained being purified by prolonged dialysis (four to six weeks) until the water outside the dialyser contained no trace of sulphates. The euglobulin fraction proved to be insoluble in distilled water, that is, it was precipitated from its solution on dialysis. For the purpose of refractometer measurements it was dissolved in a dilute salt solution and the refractive indices of the globulin solution and of a salt solution of the same concentration were separately determined. The difference between the two refractive indices afforded

¹E. Reiss *Beitr. z. chem. Physiol. u. Path.*, iv, p. 150, 1903, *Arch. f. exp. Path. u. Pharm.*, li, p. 18, 1903.

a measure of the effect of the globulin upon the refractive index of its solution. The quantity of globulin in each of the solutions employed was estimated by precipitation with alcohol and drying and weighing the precipitate thus obtained. In this way the effect due to 1 per cent of each of the proteins in question can readily be computed. The following were the results obtained by Reiss, employing solutions of the various globulins defined above

One gram of "Euglobulin" changed the refractive index of 100 cc of solution by 0.00230

One gram of "Pseudoglobulin I" changed the refractive index of 100 cc of solution by 0.00224

One gram of "Pseudoglobulin II" changed the refractive index of 100 cc of solution by 0.00230

As Reiss himself points out, the differences between these figures are within the experimental error of the estimate, and from his results we may conclude that 1 gram of any of the globulins of serum, when dissolved in 100 cc of water or dilute salt solution, changes the refractive index of the solvent by about 0.00230

In a previous communication² I have extended and confirmed the results of Reiss in so far as they apply to the "Euglobulin" or the globulin fraction which is insoluble in distilled water. I prepared euglobulin by precipitation from diluted serum with CO_2 and, after careful purification, dissolved carefully estimated amounts in measured volumes of dilute KOH. In this way I found the value of a (=change in the refractive index of the solvent due to 1 gram of protein) for this protein to be³ 0.00229 ± 0.00024

This value of a is, within the experimental error, identical with that obtained by Reiss for each of his different globulin fractions. We may therefore assume that in all probability the value of a for the globulins of serum, irrespective of the mode of precipitation, to be that which I have just cited.

In measuring the value of the constant a for the *albumins* of blood-serum, Reiss proceeded as follows

The filtrate obtained by half-saturating serum with ammonium sulphate and filtering off the precipitated globulins was acidified

² T. Brailsford Robertson, *This Journal*, viii, p. 441, 1910

³ T. Brailsford Robertson, *Die physikalische Chemie der Proteine*, Dresden, p. 323, 1912

by the cautious addition of $\frac{1}{2}$ H_2SO_4 and the precipitate which was thus produced was allowed to stand until it became crystalline ⁴

This substance after separation from the mother liquor, was redissolved and recrystallized twice and finally dissolved in distilled water and dialyzed for from four to six weeks until salt-free. The value of a for this substance ("crystalline serum-albumin") proved to be 0.00201

The mother-liquor, after the deposition of the "crystalline serum-albumin" contained another protein characterized as "amorphous serum albumin". This solution was dialyzed for from four to six weeks and the value of a for this protein was determined as in the previous cases, it proved to be 0.00183

Continuing his investigations, Reiss arrived at the remarkable conclusion that the specific refractivity of the mixed proteins in serum is actually *less* (0.00170 to 0.00175) than the specific refractivities of any of the constituent proteins of the mixture, thus rendering it impossible to estimate from the value of a for the mixed proteins in blood-serum the relative proportion of globulins and albumins contained therein. This appeared to me to be a very important conclusion, meriting further investigation, for the following reasons

I have found⁵ that that change in the physical and chemical condition of a dissolved protein which immediately precedes coagulation, and may be induced by the addition of a coagulating (dehydrating) agent to the solution, is accompanied by a decrease in the specific refractivity of the protein. Now there is much reason for believing that the proteins in circulating blood or in unaltered blood-serum are not merely present therein in the form of a mixture, but in the form of a chemical complex possessing recognizably different physical and chemical properties from those of the constituent proteins out of which it is built up ⁶. It appeared

⁴ Hofmeister *Zeitschr f physiol Chem*, xiv, p 163, 1889, xvi, p 187, 1891, A. Gürber, *Sitz d physik med Ges zu Würzburg*, p 143, 1894, cited after Schulz, *Die Krystallisation von Eiweissstoffen*, Jena, p 13, 1901. A. Michel *Verh d physik med Ges zu Würzburg*, xxv, 3, p 28, cited after Schulz *loc cit*, p 13, H. T. Krieger *Dissertation*, Strassburg, 1899, cited after Schulz *loc cit*, p 11

⁵ T. Brailsford Robertson *Die physikalische Chemie der Proteine*, Dresden, 1912, chapters 10 and 13

⁶ T. Brailsford Robertson *ibid*, pp 126-133

possible that in the building up of this complex the individual proteins composing it might sustain a loss of refractive power and that the phenomenon observed by Reiss might be susceptible of this explanation. Accordingly, the following investigations were undertaken

2 EXPERIMENTAL

In endeavoring to measure the refractivity of the mixed proteins in blood-serum itself we are confronted with the difficulty of determining the refractivity of the solvent in which these proteins are dissolved, that is, the proportion of the difference between the refractivity of the serum and that of distilled water which is due to the *non-protein* constituents of serum. For reasons which will be referred to later, I did not consider the estimates of the refractivity of the non-protein constituents of blood-serum which were adopted by Reiss to be wholly satisfactory. Accordingly I preferred to assume, until further evidence should demonstrate that assumption to be false, that the solvent in which the proteins of ox-blood serum are dissolved may be regarded, for the purposes of refractometer measurements, as being essentially $\frac{M}{100}$ sodium chloride.⁷ As we shall see, the results of my measurements show that this assumption is justified.

Fresh ox-blood serum was prepared by whipping and centrifugalizing the freshly-drawn blood.⁸ This serum was diluted with varying proportions of distilled water. The refractive indices

⁷ The refractive powers of dilute equivalent solutions of the chlorides of the mineral bases found in serum are nearly equal. Since only traces of KCl and CaCl₂ are present in serum we may safely take the refractivity of the saline constituents of serum to be that of $\frac{M}{100}$ NaCl. In assuming that the refractivity of the non-protein constituents of serum is likewise identical with that of a $\frac{M}{100}$ NaCl solution we are assuming that the fats, sugars, etc., which are normally present only in small amounts in the serum derived from systemic blood, take only a negligible part in determining the refractivity of serum.

⁸ A source of error which may possibly invalidate some estimates of the concentrations of solid constituents in blood obtained from slaughter-houses may be pointed out here. When blood is allowed to clot and the clot is left in the cold-chamber of a slaughter house to contract and express serum very considerable evaporation may occur under the condition of desiccation which is necessarily maintained in such rooms. Serum thus prepared may be found on analysis, to contain from 10 to 12 per cent of proteins.

of these solutions and of distilled water and of $\frac{N}{6}$ sodium chloride solution were measured by means of a Pulfrich refractometer at about 20° C, employing a sodium flame as the source of light

The results which were obtained are expressed in the accompanying table. The value of n_1 (=refractive index of the solvent in which the serum-proteins are dissolved) is calculated upon the assumption that the non-protein portion of serum may be regarded, for the purposes of refractometer measurements, as being essentially $\frac{N}{6}$ NaCl and upon the further assumption (which I have experimentally verified) that the difference between the refractive index of a sodium chloride solution of this or lower concentrations and that of distilled water is directly proportional to the concentration of the sodium chloride solution

TABLE 1

SOLUTION	n =REFRACTIVE INDEX OF SOLUTION	n_1 =REFRACTIVE INDEX OF SOLVENT	$n-n_1$
Distilled water	1 33410		
$\frac{N}{6}$ NaCl	1 33567	1 33410	0 00157
5 cc of serum + 20 cc of water	1 33759	1 33441	0 00318
10 cc of serum + 15 cc of water	1 34139	1 33463	0 00666
15 cc of serum + 10 cc of water	1 34511	1 33504	0 01007
20 cc of serum + 5 cc of water	1 34872	1 33536	0 01336
25 cc of serum + 0 cc of water	1 35224	1 33567	0 01657

Dividing each of the values of $n-n_1$ tabulated above by the number of cubic centimeters of serum in 25 cc of the corresponding mixture of serum and water we obtain

TABLE 2

SOLUTION	$n-n_1$ CUBIC CENTIMETERS OF SERUM IN 25 CC SOLUTION
5 cc of serum + 20 cc of water	0 00064
10 cc of serum + 15 cc of water	0 00067
15 cc of serum + 10 cc of water	0 00067
20 cc of serum + 5 cc of water	0 00067
25 cc of serum + 0 cc of water	0 00066

From the constancy of this ratio, since, of course, each cubic centimeter of serum contains the same quantity of serum-protein, we may conclude that *the value of a for the mixed serum proteins is independent of the dilution*

In order to determine, from these results, the *absolute* value of a for the mixed serum proteins it was only necessary to determine the percentage of total proteins which was contained in the serum under investigation. This determination was performed in the following manner

Two samples of the serum, measuring respectively 2.95 and 3.00 cc. were accurately delivered, drop by drop, into a small hardened nitrogen-free filter-paper (5.5 cm. diameter) which was at the same time kept filled with absolute alcohol. The filters and contained protein were then washed in alcohol and ether and dried for two or three hours at 40°. They were then analyzed for nitrogen by the Kjeldahl method. From the quantity of nitrogen thus found, the percentage of protein in the original serum was calculated on the assumption that the nitrogen-content of the serum-proteins is 15.9 per cent.⁹ The results follow

SAMPLE	N IN SAMPLE	PERCENTAGE OF PROTEINS IN SERUM
cc	mg	
2.95	39.5	8.4
3.00	41.0	8.6
Mean		8.5 ± 0.1

From this result the concentration of serum-proteins in each of the mixtures enumerated in Table I can readily be computed. We can compute the value of a from those of $n - n_1$ in Table I by adding together all of the observed values of $n - n_1$ and dividing this sum by the sum of the concentrations of protein in the mixtures employed.¹⁰

The possible error of this estimate may be computed if we recollect that each determination of the angle of total reflection is

⁹ O. Hammarsten *Arch. f. d. ges. Physiol.*, **xxii**, p. 431, 1880; E. Abderhalden *Zeitschr. f. physiol. Chem.*, **xxviii**, p. 495, 1903.

¹⁰ For the rationale of this procedure consult T. Brailsford Robertson *This Journal*, **viii**, p. 507, 1910.

liable to an error of $\pm 1'$ corresponding, for solutions of these refractivities, to an error of between ± 0.00008 and ± 0.00009 in the determination of $n - n_1$. Proceeding in this way we find that for the serum proteins dissolved in diluted or undiluted serum the value of a (=change in the refractive index of the solution due to 1 per cent of protein) is 0.00195 ± 0.00002 .

This result, it will be observed, is very different from the above-cited result obtained by Reiss ($a = 0.00172$). The value of a for the mixed proteins, instead of being less than that for any of its constituents, would appear, as might have been expected, to be intermediate in magnitude between the value of a ($= 0.00183$) for serum-albumin and that of a ($= 0.00229$) for serum globulins.

The protein-complex into which the individual proteins of unaltered serum would appear to be built up is decomposed by acids,¹¹ consequently, it appeared of importance to ascertain whether the refractivity of the mixed serum-protein is changed by the communication of an acid reaction to the serum. Accordingly, samples of the same serum as that employed in the experiments cited in Table I were diluted with $\frac{1}{16}$ hydrochloric acid instead of with distilled water. About 12.5 cc of $\frac{1}{16}$ acid suffice to communicate a neutral reaction to 100 cc of undiluted serum,¹² hence all of the mixtures of serum with $\frac{1}{16}$ HCl which were employed in these experiments were acid in reaction. The values of n_1 (=refractive index of the solvent) are calculated in the same way as those in Table I, with the aid of the further assumption (the truth of which I have experimentally verified) that the change in the refractive index of water due to the addition of HCl is proportional, within the limits of concentration considered, to the concentration of the HCl. The following were the results obtained

¹¹ W. B. Hardy, *Journ. of Physiol.*, xxxiii, p. 251, 1905 (appendix), T. Brailsford Robertson, *Die physikalische Chemie der Proteine*, Dresden, 1912, pp. 126-133.

¹² T. Brailsford Robertson, *This Journal*, vii, p. 351, 1910.

TABLE 3

SOLUTION	n =REFRACTIVE INDEX OF SOLUTION	n_1 =REFRACTIVE INDEX OF SOLVENT	$n-n_1$
Distilled water	1 33410		
$\frac{M}{5}$ NaCl	1 33567	1 33410	0 00157
$\frac{N}{10}$ HCl	1 33481	1 33410	0 00071
5 cc of serum + 20 cc of $\frac{N}{10}$ HCl	1 33815	1 33498	0 00317
10 cc of serum + 15 cc of $\frac{N}{10}$ HCl	1 34155	1 33515	0 00640
15 cc of serum + 10 cc of $\frac{N}{10}$ HCl	1 34519	1 33533	0 00986
20 cc of serum + 5 cc of $\frac{N}{10}$ HCl	1 34881	1 33550	0 01331
25 cc of serum + 0 cc of $\frac{N}{10}$ HCl	1 35224	1 33567	0 01657

Dividing each of the values of $n-n_1$ tabulated above by the number of cubic centimeters of serum in 25 cc of the corresponding mixture of serum and $\frac{N}{10}$ acid we obtain

TABLE 4

SOLUTION	$\frac{n-n_1}{\text{CUBIC CENTIMETERS OF SERUM IN 25 CC SOLUTION}}$
5 cc of serum + 20 cc $\frac{N}{10}$ HCl	0 00063
10 cc of serum + 15 cc $\frac{N}{10}$ HCl	0 00064
15 cc of serum + 10 cc $\frac{N}{10}$ HCl	0 00066
20 cc of serum + 5 cc $\frac{N}{10}$ HCl	0 00067
25 cc of serum + 0 cc $\frac{N}{10}$ HCl	0 00066

From the constancy of this ratio we may conclude that *acidification of serum does not alter the refractivity of the serum proteins*

Computing, from the results cited in Table 3, the value of a for the mixed proteins in acidified serum we obtain $a = 0 00193 \pm 0 00002$, a result which is identical within the experimental error, with that obtained for the proteins in neutral serum

In seeking for a reason for the discrepancy between my results and those cited by Reiss the initial assumption upon which my estimates of a are based, namely that the non-protein constituents of serum may, for the purpose of refractometer measurements, be regarded as $\frac{M}{5}$ NaCl, at once suggests itself as a possible source of error. Reiss' estimates of the refractivity of the non-protein constituents of serum (0 00270 to 0 00292 in different experiments)

is much higher than mine (0.00157, cf. Tables 1 and 3). If non-protein constituents other than mineral salts occur in serum in sufficient amounts to appreciably influence the refractivity of the serum, then their refractivity would be added to that of the proteins in my estimates and the values of α obtained above would be too high. Accordingly, the following experiments were undertaken.

Four samples of serum were taken of which the first (sample 1) measured 100 cc. and the remainder (samples 2, 3 and 4) 80 cc. each. The proteins from sample 1 were precipitated immediately by the addition of 10 volumes of absolute alcohol. To sample 2 were added 20 cc. of $\frac{N}{10}$ HCl and the proteins were then immediately precipitated by the addition of 10 volumes of absolute alcohol. To samples 3 and 4 were added 20 cc. of $\frac{N}{10}$ acetic acid and $\frac{N}{10}$ hydrochloric acid, respectively. These mixtures were allowed to stand at room temperature for about two hours before precipitating the proteins from them by the addition of ten volumes of absolute alcohol.

These precipitates were washed three times in the volume of absolute alcohol originally employed for the precipitation and then three times in the same volume of ether, the precipitates being allowed to settle after each washing and the supernatant fluid drawn off by means of a syphon. The thick suspensions of protein in ether which were finally obtained were dried at 40° over sulphuric acid for twenty-four hours, pulverized and sifted and then dried over sulphuric acid at room temperatures for over three weeks.

The solutions of these proteins in distilled water are not sufficiently transparent for refractometer readings. On adding a little alkali, however, they at once clear up, 1 per cent solutions being of a clear pale yellow color. Accordingly solutions were prepared in the following manner.

Three grams of each of the above preparations were separately dissolved in 300 cc. of $\frac{N}{5}$ KOH, by first stirring up the protein with a little of the solvent until it formed a paste and then adding the remainder of the solvent and stirring rapidly for about an hour. One hundred cubic centimeters of each solution were diluted to 200 cc. making 0.5 per cent solutions.

The refractive indices of these solutions were determined by means of the Pulfrich refractometer at 20° C., employing a sodium flame as the source of light.

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TABLE 5

SOLUTION	n =REFRACTIVE INDEX OF SOLUTION	a =CHANGE IN THE RE- FRACTIVE INDEX OF THE SOLVENT DUE TO 1 PER CENT OF PROTEIN
$\frac{N}{50}$ KOH	1 33426	
1 per cent Proteins of Sample 1	1 33615	0 00189 \pm 0 00011
0 5 per cent Proteins of Sample 1	1 33521	
1 per cent Proteins of Sample 2	1 33615	
0 5 per cent Proteins of Sample 2	1 33521	0 00189 \pm 0 00011
1 per cent Proteins of Sample 3	1 33615	
0 5 per cent Proteins of Sample 3	1 33521	0 00189 \pm 0 00011
1 per cent Proteins of Sample 4	1 33615	
0 5 per cent Proteins of Sample 4	1 33521	0 00189 \pm 0 00011

The values of a enumerated in the third column of this table are identical with one another and also, within the experimental error, identical with those determined above for the proteins in unaltered, diluted, or acidified serum. Now from the method of preparation it is obvious that these proteins must have been free from appreciable non-protein contamination other than, possibly, small amounts of inorganic bases¹³. We may therefore conclude (1) *That acidification of serum does not alter the refractivity of the serum proteins*, (2) *That the value of a for the serum proteins is 0.00195 ± 0.00002* , (3) *That the refractivity of the non-protein constituents of serum may be regarded, without introducing any appreciable error, as that of a $\frac{N}{50}$ sodium chloride solution*, (4) *That Reiss' estimates of the refractivity of the non-protein constituents of serum are nearly 100 per cent too high*.

His excessive estimates of the refractivity of the non-protein constituents of serum are sufficient to account for the low value of a for the mixed proteins which was obtained by Reiss. It is more difficult to assign a cause for his excessive estimate of the refractivity of the non-protein constituents of serum, since, in the articles to which I have referred, he does not specifically describe how he arrived at this estimate. From the context¹⁴ however,

¹³ Cf T Brailsford Robertson *This Journal*, vii, p 351, 1910

¹⁴ In his second paper (*Arch f exp Path u Pharm*, li, p 20, 1903) he thus describes his method of determining the value of a for the mixed proteins in horse-serum. "Eine zweite Bestimmung wurde in etwas anderer

it appears that he estimated the refractivity of the non-protein constituents of serum by removing the proteins with the aid of heat-coagulation and determining the refractivity of the protein-free fluid which was thus obtained. This procedure is obviously open to the objection that during the heating of the protein solution changes of unknown magnitude (such as hydrolyses, etc.) may occur, leading to the formation of substances not normally present in serum and affecting the refractivity of the fluid. Furthermore, it is possible that in the blood-serum substances of a mucoid or proteose-like nature occur which are not coagulable by heat.¹⁵ In estimating the value of a for "amorphous serum albumin" by Reiss' method such proteins would be present in the solution and the observed refractivity (since they would be precipitable by alcohol) of "amorphous serum albumin" would, in reality, be the sum of the refractivities of serum albumin and the mucoid or proteose. Such substances, however, would not be coagulated by heat and their refractivity would, employing Reiss' method, be estimated along with that of the non-protein constituents of serum.

I have also measured the refractivity of the serum-albumin or albumins in the filtrate obtained by adding an equal volume of saturated ammonium sulphate solution to serum and filtering off the globulins thus precipitated. In this experiment we are confronted

Weise an Pferdeblutserum vorgenommen. Eine genau abgemessene Menge desselben wurde auf etwa das zehnfache verdünnt, die ausgefallenen Globuline durch abgemessenen Zusatz von Natriumkarbonat wieder zur Lösung gebracht und aus den Brechungskoeffizienten des nativen und des verdünnten Serums unter Abrechnung der Lichtbrechung des zugefügten Natriumkarbonats der Verdünnungsgrad berechnet. Sodann wurde ausprobiert, wieviel Essigsäure einen bestimmten Quantum des verdünnten Serums zugesetzt werden musste, um das Eiweiss in der Hitze zum völligen Ausfallen zu bringen. Eine dementsprechend gestaltete Mischung wurde im zugeschmolzenen Glasröhrchen etwa 10 Minuten auf 100° erhitzt. Nach einigem Stehenlassen wurde das Röhrchen geöffnet und ein Tropfen der obenstehenden Flüssigkeit—der nur noch Spuren Eiweiss enthielt—refraktometrisch untersucht. Der Eiweissgehalt des nativen wie des verdünnten Serums wurde durch Wägung (Fällen mit Alkohol, einstündiges Erhitzen auf 80°) bestimmt. Aus den so erhaltenen Zahlen berechnete sich der Anteil des Brechungskoeffizienten für 1 Proz. Eiweiss auf 0.00175, für die Nicht-eiweisskörper des gesamten Serums auf 0.00292."

¹⁵ Cf. O. Hammarsten *Ergeb. d. Physiol.*, 1, Abt. 1, p. 354, 1902.

by the difficulty that the refractivity of serum-albumins dissolved in half-saturated ammonium sulphate may not necessarily be the same as their refractivity in distilled water or in serum, since, as I have shown elsewhere,¹⁶ the refractivity of a protein, upon successive additions of a coagulating (dehydrating) agent to its solution tends to diminish some time before manifest coagulation occurs. It occurred to me, however, that if this were the case with serum-albumin dissolved in half-saturated ammonium sulphate, then the value of α should alter upon dilution of this solution and finally approach its value in distilled water, accordingly the following experiments were undertaken

To 250 cc of fresh centrifugalized ox-serum were added 250 cc of saturated ammonium sulphate solution, and the mixture was filtered through hardened filter paper. The entire process of filtration occupied about three and one-half hours, the filter being changed from time to time as it became clogged, in order to secure as rapid filtration as possible and thus avoid concentration of the filtrate through evaporation. Samples, measuring 25, 33.3, 50, 66.7 and 75 cc, respectively, of this filtrate were diluted to 100 cc by the addition of distilled water.¹⁷ It will be observed, therefore, that the proteins in the serum were diluted, in each mixture, to exactly the same extent as the saturated ammonium sulphate solution.

To 250 cc of distilled water were similarly added 250 cc of saturated ammonium sulphate and samples measuring 25, 33.3, 50, 66.7, and 75 cc of this mixture were similarly diluted to 100 cc.

The refractive indices of these serum and ammonium sulphate solutions were measured at 24° C in a Pulfrich refractometer, employing a sodium flame as the source of light. The following were the results obtained

¹⁶ Cf. Previous communications of this series, *This Journal*, also *Die physikalische Chemie der Proteine*," Dresden, 1912, chapter 13.

¹⁷ It is necessary thus to specify exactly the methods of dilution employed, on account of the volume-change which occurs on diluting strong ammonium-sulphate solutions.

TABLE 6.

SOLUTION	n	$n-n_1$	$(n-n_1) \times \text{DILUTION OF THE ORIGINAL SERUM}$
Distilled water	1 33364		
$\frac{1}{2}$ Saturated Am SO ₄	1 34419		
Seralbumins dissolved in above	1 34553	0 00134 \pm 0 00008	0 01072 \pm 0 00064
$\frac{1}{2}$ Saturated Am SO ₄	1 34753		
Seralbumins dissolved in above	1 34940	0 00187 \pm 0 00009	0 01122 \pm 0 00054
$\frac{1}{2}$ Saturated Am SO ₄	1 35388		
Seralbumins dissolved in above	1 35667	0 00279 \pm 0 00009	0 01116 \pm 0 00036
$\frac{1}{2}$ Saturated Am SO ₄	1 35993		
Seralbumins dissolved in above	1 36369	0 00376 \pm 0 00009	0 01128 \pm 0 00028
$\frac{1}{2}$ Saturated Am SO ₄	1 36279		
Seralbumins dissolved in above	1 36695	0 00416 \pm 0 00009	0 01110 \pm 0 00024
$\frac{1}{2}$ Saturated Am SO ₄	1 37098		
Seralbumins dissolved in above	1 37581	0 00483 \pm 0 00009	0 00966 \pm 0 00018

The average of the first five values of the product $(n-n_1) \times$ dilution of the original serum is $0\ 01110 \pm 0\ 00042$, all of them being identical within this experimental error, while the value of this product for the serum albumins dissolved in one-half saturated ammonium sulphate is considerably less. We may, therefore conclude that the refractivity of the serum albumins dissolved in three-eighths saturated or more dilute solutions of ammonium sulphate is independent of the dilution, and, consequently, that the value of a for serum albumins dissolved in three-eighths saturated or more dilute solutions of ammonium sulphate is the same as its value for serum albumins dissolved in distilled water or in serum.

The total refractivity of the serum albumins plus the non-protein constituents in the ox-serum employed is, therefore, $0\ 01110 \pm 0\ 00042$.

The total refractivity of the proteins plus the non-protein constituents in ox-serum¹⁸ is $0\ 01815 \pm 0\ 00017$.

¹⁸ Cf. the value for a for the mixed proteins of serum and the value of $n-n_1$ for $\frac{M}{V}$ NaCl in Table 1.

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The difference between these two estimates is the total refractivity of the globulins in serum, namely 0.00705 ± 0.00030

Hence the percentage of globulins in ox-serum¹⁹ is

$$\frac{705 \pm 30}{229} = 3.1 \pm 0.1$$

The total concentration of proteins in the ox-serum employed was, as we have seen, 8.5 ± 0.1 . Hence the concentration of the albumins was $8.5 \pm 0.1 - 3.1 \pm 0.1 = 5.4 \pm 0.1$

The total refractivity of the serum albumin plus the non-protein constituents of the ox-serum employed was, as we have seen 0.01110 ± 0.00042

Hence the total refractivity of the serum albumins alone was $0.01110 \pm 0.00042 - 0.00157 = 0.00953 \pm 0.00042$

Hence the value of a for the serum albumins is

$$\frac{0.00953 \pm 0.00042}{5.4 \pm 0.1} = 0.00177 \pm 0.00008$$

an estimate which is, within the experimental error, *identical with Reiss' estimate of a for "amorphous serum albumin"*²⁰

This result is very striking, for Reiss, by adding acid to his ammonium sulphate solution of the albumins of *horse-serum* and allowing the precipitate thus produced to stand, was able to separate the serum albumins into two fractions, the one, the "crystalline" fraction possessing a much higher value of a ($= 0.00201$) than the other, the "amorphous" fraction (0.00183). Yet my estimate of the value of a for the mixed albumins is identical with or even slightly lower than Reiss' estimate of the refractivity of the "amorphous" fraction²¹. It would appear, therefore, that crystallizable serum-albumin does not exist in appreciable quantity in ox-serum, a conclusion which finds striking confirmation in the fact that Krieger²² was unable to obtain crystalline albumin from ox-serum.

¹⁹ Cf. estimate of a for serum-globulins in Introduction

²⁰ Cf. Introduction

²¹ Reiss himself states that his preparation of "amorphous" serum albumin was probably contaminated with "crystalline" serum-albumin

²² H. T. Krieger, Dissertation, Strassburg, 1899, cited after *Maly's Jahresbericht*, p. 14, 1899

From the above data we may conclude that the percentages of the globulins and albumins in the α -serum employed were as follows

Total globulins	31 ± 0.1 per cent
Total albumins	54 ± 0.1 per cent

I have also determined the percentage of the "insoluble" or CO_2 -precipitable globulin (euglobulin) in α -serum in the following way

Three samples of fresh centrifugalized α -blood serum, measuring 100 cc each, were diluted with distilled water to 1000 cc and CO_2 was bubbled through the mixtures at a quick rate. The time of passage of the CO_2 was purposely varied among the three samples, the least time of passage being 1 hour and the longest 2 hours. The precipitate of insoluble globulin which was thus produced was allowed to settle in tall glass cylinders and the supernatant fluid drawn off by means of a syphon. The precipitate was then washed in a liter of distilled water, this washing was repeated.²¹ The subnatant precipitates were then dissolved by the addition of 10 cc of $\frac{N}{10}$ KOH and the volumes made up in each case to 100 cc. The refractive indices of these solutions were measured in the usual manner with the following results

TABLE 7

SOLUTION	n	$n - n_1$	PER CENT OF GLOBULIN ($d = 0.00229$)
$\frac{N}{100}$ KOH	1 33387		
1	1 33560	0.00173 ± 0.00008	} 0.76 ± 0.04
2	1 33560	0.00173 ± 0.00008	
3	1 33560	0.00173 ± 0.00008	

Hence the percentages of the various proteins contained in the α -serum employed were as follows

²¹ The error introduced by contamination with the other proteins of serum must, after this washing, have been negligible, as may readily be calculated in the following way. The total refractivity of the substances dissolved in serum is 0.01815. The volume of the subnatant suspensions obtained in the above processes of washing was always less than 100 cc, hence after dilution and two washings the refractivity of the dissolved substances in the fluid in which the CO_2 globulin was suspended must have been less than $1/1000$ of 0.01815, that is, less than 0.00002. The experimental error in the determination of the refractivity is ± 0.00008 .

"Insoluble" globulins	0.76±0.04
"Solubblue" globulins	2.34±0.10
Total albumins	5.40±0.10
Total	8.50±0.10

3 DISCUSSION OF THE RESULTS

The quantitative estimates, just enumerated, of the percentages of the various proteins of ox-serum are not in very good agreement with the analytical data published by previous observers. Neither do the data obtained by previous observers agree among themselves.²⁴ This is usually attributed to individual variability among the animals investigated. I have not as yet carried out experiments with the view to ascertaining to what extent the protein content of the sera of different individuals of the same species varies when determined by the methods employed in this investigation, but I am inclined to think that the content of proteins in the sera of a given species is not nearly so variable as investigators have been inclined to imagine. Thus I have determined the percentage of "insoluble" globulin in two samples of ox-serum obtained at different times, and found the same percentage of this protein in each sample.²⁵ I have also determined the refractivities of the filtrates obtained from several distinct samples of ox-serum after one-half saturation with ammonium sulphate and filtering off the precipitated globulins, and I have always found the refractivities of these filtrates to be the same within the experi-

²⁴ Cf. the results for human blood serum obtained by O. Hammarsten and F. A. Hoffmann, cited by F. A. Hoffmann *Arch f exp Path u Pharm*, **vi**, p. 133, 1883.

²⁵ Hammarsten (*Arch f d ges Physiol*, **vii**, p. 413, 1878) found a much higher content of CO₂-precipitable globulin in ox-serum than I, namely, from 0.83 per cent to 1.18 per cent. He also finds the quantity of globulin which is precipitated by dialysis considerably greater (1.13 per cent to 1.69 per cent) than that which is precipitated by CO₂. Quinan (*Univ of California Publications, Pathol*, **i**, p. 1, 1903) on the contrary, working with goat-serum, finds that the percentage of protein which is precipitated by dialysis is exactly the same as that which is precipitable by dilution and the passage of CO₂. Curiously enough, also, he found the content of CO₂-precipitable globulin in goat sera to be exactly the same as that which I have found in ox-sera, namely, 0.76 per cent.

mental error While my results are not as yet sufficiently extended in this direction, therefore, to warrant a definite statement that the content of proteins in α -sera is only slightly variable, yet they are such as to cast suspicion upon the validity of the highly variable figures obtained by other methods of determination

The most marked deviation between my results and those of other observers is in the relative proportion of globulin and albumins in α -serum According to Hammarsten (*loc cit*) globulins are present in α -serum in excess of albumins in the (variable) proportion of 1.0842

The majority of investigators have employed horse or human sera in their experiments and, consequently, analytical data regarding the proteins in α -serum are not very numerous Nevertheless, two possible sources of the divergence between my results and those of Hammarsten may be tentatively suggested here

First It has been pointed out by Porges and Spiro²⁶ that serum obtained from blood by the shrinkage of the clot contains a considerably higher percentage of substances resembling "insoluble" globulin than fresh, whipped and centrifugalized serum The origin of this globulin is not clear, but the results of Spiro and Porges are sufficient to indicate that data obtained with serum slowly expressed from clotted blood are not to be relied upon as indications of the actual conditions in circulating blood

Second Hammarsten employed saturated magnesium sulphate to precipitate the globulins of sera, whereas I have employed half-saturated ammonium sulphate There are indications, according to some observers²⁷ that saturated magnesium sulphate precipitates a proportion of albumin as well as globulin

Objections have been made by several observers notably Wiener²⁸ to the use of ammonium sulphate as a precipitant of globulins from serum According to this investigator, unless the serum be considerably diluted before the ammonium sulphate is added, some other substance besides globulin is precipitated, for on successive dilution of serum and half-saturation with ammonium sulphate,

²⁶ O. Porges and K. Spiro *Beitr. z. chem. Physiol. u. Pathol.*, **III**, p. 277, 1902

²⁷ Hevnsius *Arch. f. d. ges. Physiol.*, **XXIV**, p. 330, 1884; E. Marcus *Zeitschr. f. physiol. Chem.*, **XXVIII**, p. 559, 1899

²⁸ H. Wiener *Zeitschr. f. physiol. Chem.*, **LXXV**, p. 29, 1911

less and less protein is precipitated until a minimum (about 80 per cent of the quantity obtained from undiluted serum) is obtained

According to Kauder,²⁹ on the other hand, complete precipitation of the serum globulin is attained somewhat prior to 50 per cent saturation and a very considerably higher percentage of ammonium sulphate must be added before precipitation of the albumins begins. Haslam,³⁰ again, believes that one-half saturation with ammonium sulphate does not remove all of the globulins from serum, since if the globulins are removed from serum by half-saturation with ammonium sulphate and further ammonium sulphate be added to the clear filtrate until precipitation just begins, this second precipitate, after filtering off and redissolving in water is now found to be precipitable by one-half saturation with ammonium sulphate. It appears to me, however, that this conclusion of Haslam's is vitiated by the tacit assumption that the action of dehydrating agents upon albumins is reversible, *i e*, that albumin after exposure to concentrated ammonium sulphate is unaltered in its properties. It is a familiar fact that extreme dehydration may in many instances induce irreversible changes in proteins, *i e*, "denaturation," while the results of Starke and Mott³¹ would appear to indicate that dehydrating agents (*e g*, heat) may convert serum albumins into substances which resemble globulin in their behavior.

It is, of course, impossible to establish the chemical individuality of a substance merely by its precipitability with this reagent, or with that, and the methods of estimating serum-proteins which have hitherto been employed suffer from that fact that the means of separating the protein are also employed as means of identifying them. While I have not, as yet, tested the validity of the objections urged by Wiener and Haslam against the use of ammonium sulphate as a precipitant of globulins by the new method of determination, yet the method which I am about to outline, based upon the results enumerated in the experimental part of this paper, has the following advantages over the methods at present in use

²⁹ G. Kauder *Arch f exp Path u Pharm*, **11**, p 411, 1886

³⁰ H. C. Haslam *Journ of Physiol*, **111**, p 267, 1904

³¹ J. Starke *Zeitschr f Biol*, **11**, p 419, 1900, L. Mott *Beitr z chem Physiol u Path*, **14**, p 563, 1904

1 The method is extremely rapid and requires very little manipulation. Apart from the Kjeldahl estimation, which need only be employed when the presence of "crystalline" serum albumin is suspected, and from the time required for the settling of the "insoluble" globulin precipitate, a period of two hours suffices for several simultaneous determinations.

2 The experimental errors of the method are not greater than those of the methods now in use and they can be quantitatively estimated with great exactitude.

3 Whether the precipitate which is produced in serum by one-half saturation with ammonium sulphate comprises all of the globulins or not, the constancy of the value of a for the substance thus precipitated and for the substance left in solution shows that it is either pure globulin or a mixture of perfectly definite and invariable composition, provided the conditions of precipitation are strictly adhered to. If the proportion of this substance is different in the serum of different individuals or species, we may be fairly confident, therefore, that the quantitative relations of the globulin and albumin groups are different in these animals.

4 The substances estimated are defined by a definite and readily measurable physical property and not merely by their precipitability by the reagents employed to isolate them.

4 APPLICATION OF THE RESULTS TO THE QUANTITATIVE DETERMINATION OF THE VARIOUS PROTEINS IN BLOOD-SERA

Owing, as I have explained in the experimental part of this paper, to the fact that his estimates of the refractivity of the non-protein constituents of blood-serum were too high, Reiss' refractometric method of estimating proteins could only be applied to blood-sera for the purpose of estimating the total protein content, and these estimates were vitiated by the same error. The discovery that the refractivity of the mixed (or combined) proteins in sera is the sum of the refractivities of its parts renders the method, with modifications, available for the separate determination of each of the proteins at present known with certainty to exist in blood-sera. The following is an outline of the method which I propose.

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1 An accurately measured volume of fresh whipped and centrifugalized serum is diluted to ten times its volume with distilled water. Carbon dioxide is bubbled through this solution in a tall glass cylinder at a good rate (two or three bubbles per second) for at least one hour, preferably two hours. The precipitate thus obtained is washed twice with water, using each time ten times the original volume of the serum. To the final suspension of globulin thus obtained sufficient $\frac{N}{10}$ KOH is added to render the solution, after dilution to the volume originally occupied by the serum, one hundredth normal. The solution thus obtained is diluted to the original volume of the serum and its refractive index and that of $\frac{N}{100}$ KOH are determined at the same temperature. The difference between the two readings, divided by 0.00229, yields the percentage of "insoluble" globulin in the original serum.

2 To an accurately measured volume of the same serum is added an equal volume of saturated ammonium sulphate solution and the globulins thus precipitated are filtered off, the filtrate is collected and diluted to one-half with water and the refractive index of the mixture thus obtained is measured. At the same time we measure the refractive index of a one-fourth saturated solution of ammonium sulphate prepared by adding to a portion of the saturated ammonium sulphate solution an equal volume of distilled water and diluting the solution thus obtained to one-half. The difference between these two readings, multiplied by 4 and diminished by 0.00157 (the refractivity of the non-protein constituents of serum), yields the total refractivity of the albumins of the serum. If crystallizable albumin be absent, then this figure, divided by 0.00177, yields the percentage of albumin in the original serum.

3 The refractive index of the original serum is determined and that of $\frac{N}{8}$ NaCl. The difference between the two readings yields the total refractivity of the proteins in the serum. Subtracting the refractivity of the albumins, determined above, we obtain the total refractivity of the globulins in the serum. This estimate, divided by 0.00229, yields the total percentage of globulins in the serum.

4 The sum of the percentages of albumin and globulin yields the total percentage of proteins in the serum. If desired, this estimate may be checked by a Kjeldahl determination of the nitrogen in the serum employed.

If crystallizable serum albumin is present, then a Kjeldahl determination of the nitrogen in the original serum is rendered necessary.² From this the total percentage of protein in the serum is estimated. The refractivity of the total proteins is estimated as in 3 and from it is subtracted the refractivity of the total albumins, determined as in 2. The difference, divided by 0.00229, yields the percentage of globulins. Subtracting this from the percentage of mixed proteins, we obtain the percentage of mixed albumins. From this and the refractivity of the albumins we estimate the value of a (=change in the refractivity of a solution due to 1 per cent of the protein) for the mixed albumins. From the value of a for crystalline serum-albumin ($=0.00201$), according to Reiss and that of a for amorphous serum-albumin ($=0.00177$) we estimate the proportion of crystallizable to amorphous albumin in the serum.

It will be seen, on referring to the tabulated results in the experimental paper that had the method outlined been employed instead of the more extensive measurements enumerated in the experimental part, results would have been obtained identical with those cited therein.

5 CONCLUSIONS

1 The value of a (=change in refractive index of a solvent caused by the solution of 1 gram of protein) for the mixed proteins of ox-serum is the same whether the proteins are dissolved in the native serum, or precipitated by alcohol, washed in alcohol, and ether, and dried and dissolved in 10% KOH. It is also independent of the dilution and is not altered by acidification of the serum. In my experiments the value of this constant for the proteins of ox-serum was 0.00195 ± 0.00002 .

2 Reiss' estimates of the refractivity of the non-protein constituents of serum are too high. For this reason, his conclusion that the refractivity of the mixed proteins of sera is less than the sum of the refractivities of the constituent proteins is erroneous. My results indicate that the refractivity of the mixed proteins of

² According to Krieger (cited after Maly's *Jahresbericht*, xxix, p. 14, 1899) crystallizable serum albumin is not found in the sera of man, oxen, pigs, dogs, rabbits, or fowls.

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ox-serum is equal to the sum of the refractivities of the separate constituent proteins

3 For refractometric purposes the non-protein constituents of serum may be regarded as being, substantially, $\frac{1}{2}$ sodium chloride

4 The value of a for the albumins of ox-serum dissolved in three-eighths saturated or more dilute solutions of ammonium sulphate is identical with its value in distilled water I find it to be 0.00177 ± 0.00008 The value of a for the albumins of ox-serum dissolved in one-half saturated ammonium sulphate solutions is somewhat lower

5 Ox-serum does not contain the crystallizable albumin which is found in horse-serum

6 The percentages of the various proteins in ox-serum have been determined refractometrically with the following results

	Per cent
"Insoluble" globulins	0.76 ± 0.04
"Soluble" globulins	2.34 ± 0.10
Total globulins	3.1 ± 0.1
Total albumins	5.4 ± 0.1
	<hr/>
Total proteins	8.5 ± 0.1

7 Reasons are given for preferring the refractometric method to the methods at present in use for the analysis of serum-proteins

8 A method of procedure for the refractometric analysis of serum proteins is outlined in detail

QUANTITATIVE DETERMINATION OF BENZOIC, HIP- PURIC, AND PHENACETURIC ACIDS IN URINE¹

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INTRODUCTION

Since 1824 when Wöhler first called attention to the fact that the formation of hippuric acid represented a synthetic reaction by the animal body, this substance has occupied an interesting place in biological chemistry. In recent years it has received special attention from the fact that the study of its production may throw light upon certain phases of nitrogen metabolism. However, owing to the difficulty of its quantitative estimation, the progress of such work has been slow.

Bunge and Schmiedeberg² first developed a method which with modifications has been in use in various laboratories in preference to all others. While the method, in that it aims to isolate the acid in pure form and to weigh it as such, is a desirable one, it still leaves much to be desired because of its difficulty of manipulation, its tediousness, and its lack of accuracy. This is very evident by the far from simple modifications that have been suggested from time to time.³

F. Blumenthal⁴ sought to avoid the error, incident to the impossibility of crystallizing the hippuric acid quantitatively from urine, by determining the nitrogen in the residue. He did this on the

¹ Published with the permission of the Director of the Agricultural Experiment Station.

² *Archiv f. exp. Path. u. Pharm.*, vi, p. 233, 1877, xv, p. 378, 1881.

³ Dakin, *This Journal*, vii, p. 106, 1910, Ranger *ibid.*, x, p. 328, 1911.

⁴ *Maly's Jahresbericht*, xxx, p. 363, 1910.

supposition that the impurities were not nitrogen containing substances Henriques and Sorensen⁵ used the formol titration on the glycocholate liberated by boiling the hippuric acid from an ethyl acetate extract with concentrated hydrochloric acid

R Cohn⁶ decomposed the hippuric acid from an ethyl acetate extract of the evaporated urine by boiling under a reflux for five hours with concentrated HCl The liberated benzoic acid was weighed as such upon volatilization of an ether extract in a tared beaker Pfeiffer, Bloch and Reicke⁷ decomposed the hippuric acid by long continued distillation with H₂SO₄, and finally titrated the benzoic acid in the distillate Jaarsveld and Stokvis⁸ decomposed the hippuric acid, extracted with ethyl acetate, by boiling with strong NaOH The benzoic acid was shaken out with petroleum ether, and weighed after volatilization of the ether at room temperature

W Wiechowski⁹ sought to purify the benzoic acid, obtained by boiling hippuric acid with NaOH, by a steam distillation The distillate was made alkaline, evaporated to a small volume, acidified, and then extracted with petroleum ether

Without going into individual criticisms of these various methods, the use of Bunge and Schmiedeberg's method in preference to all others shows the unsatisfactory manner in which their details have been worked out Jaarsveld and Stokvis admit that they were unable to obtain a pure benzoic acid and while Wiechowski secured a purer product by steam distillation, it is practically an impossibility to steam distill benzoic acid quantitatively The determination of hippuric acid as benzoic acid by sublimation seemed to offer the most satisfactory plan for further experimentation To make this applicable to urine it was necessary to take into consideration, (a) the occurrence in urine of non-conjugated benzoic acid (benzoates), (b) the occurrence in urine of conjugated benzoic acids, which, like hippuric, yield benzoic acid in the method of decomposition adopted, (c) the decomposition of hippuric acid and its quantitative recovery as benzoic acid

⁵ *Zeitschr f physiol Chem*, lxxiii, p 327

⁶ *Festschrift für Jaffe* Braunschweig, iii, p 327, 1901

⁷ *Mal'y's Jahresbericht*, lxxvi, p 364, 1903

⁸ *Archiv f exp Path u Pharm*, x, p 71, 1879

⁹ *Hofmeister's Beiträge*, vii, p 265, 1906

Non-conjugated benzoic acid in fresh and apparently normal urines has been reported from time to time, but apparently this varies with different animals¹⁰ Jaarsveld and Stokvis¹¹ were unable to find any in normal human urine, which observation has been verified by Dakin,¹² Lewinski,¹³ and Seo¹⁴ Lewinski, however, noted exceptions upon the administration of large amounts of sodium benzoate Brugsch and co-workers,¹⁵ on the other hand, report finding free benzoic acid in dog urines on feeding sodium benzoate Apparently then, until the occurrence of non-conjugated benzoic acid has been more thoroughly investigated, in a determination of total benzoic acid in urine as hippuric acid a preliminary test for free benzoic acid should first be made Of special significance in this connection is the observation of Seo,¹⁶ that, in urine, on standing, decomposition of hippuric acid rapidly sets in, due to bacterial action Schmiedeberg¹⁷ and Minkowski¹⁸ showed that the same decomposition could be caused by an enzyme, histozyne, occurring in the kidneys of dogs and pigs If it is true that the free benzoic acid in urines originates from a decomposition of the hippuric acid, subsequent to its excretion by the kidneys, it would be permissible to calculate it as hippuric acid

Very few benzoic acid complexes besides hippuric acid are known to occur in urine On feeding very large amounts of benzoic acid to animals, apparently exceeding their synthetic capacity to form hippuric acid, urines have been observed to rotate polarized light to the right instead of to the left, and to possess strong reducing power This has been shown by Magnus-Levy¹⁹ to be due to the presence of benzoyl glycuronic acid which he was able to detect in sheep urines only when very large amounts of benzoic acid were fed With rabbits and dogs, a still smaller

¹⁰ Hammarsten's *Physiological Chemistry*, p 687, 1911

¹¹ *Archiv f exp Path u Pharm*, v, p 278, 1879

¹² *This Journal*, vii, p 103, 1909

¹³ *Archiv f exp Path u Pharm*, lvi, p 88, 1909

¹⁴ *Ibid*, lviii, p 440, 1908

¹⁵ *Zeitschr f exp Path u Therapie*, iii, p 663, 1906, v, p 731, 1909

¹⁶ *Loc cit*

¹⁷ *Archiv f exp Path u Pharm*, xiv, p 379, 1881

¹⁸ *Ibid*, xvii p 453, 1883

¹⁹ *Biochem Zeitschr*, vi, p 502, 1907

tendency to form this acid was observed Dakin²⁰ was able to find traces of it in human urine when administering 10 grams of sodium benzoate When present, it is readily detected in urines by its reducing power, its non-fermentability, and its dextro-rotation In such abnormal urines a method based on the determination of conjugated benzoic acid as hippuric is not applicable Ornithuric acid discovered by Jaffé²¹ on feeding benzoic acid to birds need not be considered here as it has not yet been isolated, so far as the writer is aware, from the urines of mammals

EXPERIMENTAL

Benzoic acid was determined essentially according to the method proposed by Dakin²² for qualitative work Steam distillation was attempted but inasmuch as the solubility, as well as the boiling point of a substance, is a factor of its volatility, it was practically an impossibility to quantitatively distill the benzoic acid, even when as much as 4 to 5 liters of distillate were collected, only 90 per cent of the total benzoic acid was accounted for On decreasing the solubility of the acid by means of phosphoric acid, a very weak acid as judged by its ability to saponify esters, slight decomposition of the hippuric acid resulted Direct extraction of the benzoic acid by means of petroleum ether was also attempted, but finally abandoned in favor of benzol Two hundred cubic centimeters of urine (cow urines only were used in all experimental work) acidified with phosphoric acid were extracted for twelve hours with 90 per cent benzol (purified by washing with dilute alkali and then with water and redistilling) By shaking the benzol solution with dilute alkali, all benzoic and hippuric acids in solution were taken up After neutralizing, the water extract was evaporated to dryness, taken up with 25 cc of water, transferred to a separatory funnel, a few grams of sodium chloride added, and shaken out with freshly distilled petroleum ether (B P, 40°) Though benzoic acid is much more soluble in ethyl ether, petroleum ether was used since this does not dissolve the traces of hippuric acid (about 0.013 gram) extracted by the benzol One hundred

²⁰ *Loc cit*

²¹ *Ber d deutsch chem Gesell*, \ and \

²² Dakin *This Journal*, vii, p 107, 1909

and fifty cubic centimeters used in five portions* were found sufficient for 0.2 gram of benzoic acid. The extracted water solution which contains hippuric acid, was added to the benzol extracted urine on which a conjugated benzoic acid determination could then be made. The petroleum ether extracts were united in a separatory funnel and allowed to stand till all traces of water in emulsified form could be separated. By running the ether, drop by drop, into a U-tube, through which a current of dry air was drawn with a suction pump, the ether was rapidly volatilized without any condensation of water. To facilitate the volatilization, which otherwise would be retarded by the cooling of the tube, it was immersed in a water bath at approximately 40° . At this temperature no appreciable loss of benzoic acid results in the time necessary for the complete volatilization of the ether and the drying of the residual benzoic acid. When thoroughly dry, the benzoic acid was purified by sublimation. The U-tube was suspended in an air oven, one arm connected with a drying bottle containing H_2SO_4 , and the other with a tared condensing tube. The condensing tube consisted of a light glass tube (20 grams) 25 cm long and 9 mm bore with bulbs 3 cm in diameter blown at 9 mm intervals. These bulbs were filled with glass wool, which proved to be very efficient in condensing the benzoic acid from the current of air drawn through the apparatus by means of a suction pump. The large bore of the condenser made it possible to make connections with the arm of the U-tube inside the air bath by means of a small cork, thus preventing condensation and a clogging of the apparatus at the point of connection. By gradually bringing the air bath up to 130° the benzoic acid was quantitatively sublimed into the condenser, which at the end of the operation, lasting usually about one hour, was cooled in a desiccator and weighed.

By the above operation no benzoic acid was recovered in any of the cow urines examined, thus confirming the findings of others²³ for human urines. That this was not due to faulty operations was shown by recovering 0.195 gram of 0.200 gram of benzoic acid which had been added to 200 cc of urine.

With the absence of non-conjugated benzoic acid in cow urines which furthermore do not reduce Fehling's solution, it seemed en-

* Dakin, Lewinski, Seo *Loc cit*

tirely permissible to determine hippuric acid as benzoic if any practical methods of decomposition were found to be quantitative. Decomposition by boiling with HCl or H₂SO₄ were not only found difficult but liable to losses of benzoic acid by volatilization. Boiling with strong alkali was found much more efficacious.

One gram of hippuric acid was boiled for two hours with 50 cc of 10 per cent NaOH under a reflux in a 500 cc Jena Florence flask. After cooling, the solution was transferred to a separatory funnel, acidified with 50 per cent H₂SO₄ and shaken out consecutively with 50, 40, 20, 20, 20 cc portions of ethyl ether. The ether was volatilized and the residual benzoic acid sublimed as previously outlined. Benzoic acid $\times 1.467$ = hippuric acid.

	HIPPURIC ACID WEIGHED OUT	BENZOIC ACID RECOVERED	HIPPURIC ACID ACCOUNTED FOR	PER CENT HIPPURIC ACID ACCOUNTED FOR
	gram	gram	gram	
A	1	0.677	0.9931	99.31
B	1	0.676	0.9916	99.16

The results prove beyond a doubt that the decomposition and recovery are quantitative. In urines, however, there are interfering substances which necessitated a procedure as subsequently outlined.

In a 500 cc flask 100 cc of urine were boiled for two hours over a low flame with 10 grams of NaOH, adding 25 cc H₂O₂, a few cubic centimeters at a time, to oxidize coloring matters. After cooling, the solution was transferred to a 200 cc volumetric flask, and slightly acidified to litmus with 50 per cent H₂SO₄. Bromine water was then added to a slight bromine odor, the solution made up to volume and filtered through a dry filter. Fifty cubic centimeters of the clear filtrate after acidification were shaken out with ether and sublimed as previously outlined, taking special precautions not to raise the temperature above 130°. High temperature may cause destructive distillation of some of the impurities extracted with the benzoic acid. In many cases sublimation at a low temperature may be facilitated by tilting the U-tube and thereby distributing the benzoic acid over a larger area. By the hydrogen peroxide treatment the coloring matters of all urines examined were readily oxidized to a pale straw color without loss or oxida-

tion of benzoic acid Dakin²⁴ in a practically neutral solution was able to oxidize benzoic acid to oxybenzoic acids by means of hydrogen peroxide That these reactions did not proceed with the solution strongly alkaline was shown by negative tests for oxybenzoic acids with ferric chloride and with Millon's reagent The bromine water was very efficient in precipitating phenols which otherwise would sublime with the benzoic acid Strong acidity at this point should be avoided as it will cause the precipitation of benzoic acid which would be lost on filtering Examples of typical results obtained are given in the following table which is supplemented with titration values for benzoic acid obtained by titrating the sublimate with $\frac{N}{20}$ NaOH using phenolphthalein as indicator

NUMBER OF URINE	BENZOIC IN FORM OF HIPPURIC ADDED	SUBLIMATE OBTAINED	SUBLIMATE CALCULATED AS HIPPURIC ACID	TITRATION VALUES FOR BENZOIC ACID	PER CENT OF ADDED HIPPURIC RECOVERED
	gram	gram	gram	gram	
I	{	0 1922	0 2821	0 1913	100 6
		0 1938	0 2844	0 1931	
		0 2016	0 2959	0 2009	
II	{	0 2036	0 2988	0 2015	
		0 170	0 3739	0 5488	
				0 3712	

With the above examples as a type there can be no doubt as to the applicability of the method to cow urines Under pathological conditions and with urines from other sources no statements can as yet be made though no serious difficulty is expected

From the table it is seen that the titration values agree remarkably well with those corresponding for pure benzoic acid This practically excludes the possibility of the presence of any sublimable homologues of benzoic acid, furthermore, the melting points of sublimate were found to correspond exactly to that of benzoic acid (121 5°)

That homologues of benzoic acid do occur in urine was shown by Salkowski,²⁵ who isolated 0 8 gram of phenaceturic acid from a liter of horse urine Phenaceturic acid would, in the method of decomposition adopted for hippuric acid, yield phenylacetic acid which like benzoic acid is readily sublimable The observa-

²⁴ This Journal, III, p 419 1907

²⁵ Ber d deutsch chem Gesell, LVII, p 3010, 1884

tions on cow urines are entirely out of harmony with those of Vasiliu²⁶ who, from the examination of the urines of sheep, comes to the conclusion that phenaceturic acid is almost as important a constituent of the urine of herbivora as hippuric acid

The lowering of titration values of the sublimate from that calculated for benzoic acid suggests the possibility of determining phenaceturic acid as well as hippuric acid. Other homologues of benzoic acid due to a longer side chain are not liable to occur in urine as it has been shown that phenyl propionic acid is oxidized in the body to benzoic acid. Phenylacetic acid differs in molecular weight from benzoic acid by one CH_2 group, therefore, any lowering of titration values can be calculated directly back to percentages of phenylacetic acid as their combined weights are known

$$\left\{ \begin{array}{l} \text{Wt of sublimate} - (\text{cc } \frac{N}{20} \text{ NaOH} \times \\ \text{gms benzoic acid in 1 cc } \frac{N}{20} \text{ solution}) \end{array} \right\} \times \text{CH}_2 \cdot \text{C}_6\text{H}_5\text{CH} \cdot \text{COOH}$$

$x = \text{weight of phenyl acetic acid}$
 Weight of sublimate $- x = \text{weight of benzoic acid}$

To test the applicability of the method 1 gram of phenaceturic acid was added to 100 cc of urine, of which the hippuric acid content was known, and the general method outlined for hippuric acid followed. The table shows the results obtained on an aliquot corresponding to 25 cc of urine and 0.25 gram added phenaceturic acid

	WEIGHT OF SUBLIMATE	TITRATION OF SUBLIMATE IN CUBIC CENTI- METERS $\frac{N}{20}$ NaOH	WEIGHT BENZOIC ACID FROM HIPPURIC ACID	WEIGHT OF PHENYL- ACETIC ACID FROM PHEN- ACETURIC ACID	WEIGHT OF BENZOIC ACID BY TITRATION	WEIGHT OF PHENYL ACETIC ACID BY TITRATION
A	0.3726	58.15	0.2030	0.1761	0.1988	0.1738
B	0.3700	57.75	0.2030	0.1761	0.1981	0.1719

While the above values agree remarkably well it must be remembered that the method is not without its limitations. The difference in molecular weight between benzoic and phenyl acetic acids is small and any impurities in the sublimate will materially affect the final values, a variation of 0.1 cc of $\frac{N}{20}$ NaOH will mean a difference of 0.0068 gram of phenylacetic acid or, when multiplied

by the conversion factor 1.4191, 0.0096 gram of phenaceturic acid. However, it is believed that this method for phenaceturic acid is far superior to fractional crystallization which up to the present time has been the only one in use. Where examination for unconjugated benzoic acid in the urine is to be made the method outlined was found entirely applicable, since phenaceturic like hippuric acid is practically insoluble in benzol.

SUMMARY

Dakin's method for isolating benzoic acid was found to yield quantitative results when followed by sublimation.

Hippuric acid and phenaceturic acid occurring together can be determined respectively as benzoic and phenylacetic acid by sublimation followed by titration.

No salts of non-conjugated benzoic acid or of phenaceturic acid were found in cow urines.

NOTE ON A CASE OF PENTOSURIA PRESENTING UNUSUAL FEATURES

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Since pentosuria was first recognized by Salkowski in 1892, upwards of thirty cases have been described. The general features of the cases have been quite similar and may be summed up briefly as follows. The majority of the cases have been met with among the Jewish races. There has usually been no marked general disturbance, and the abnormality has often been discovered by accident or when the patient has been under treatment for diabetes owing to faulty diagnosis. Examination of the urine has shown that it possesses reducing properties. With Fehling's solution reduction takes place after a somewhat long latent period (about half a minute), the solution turns yellow, no cuprous oxide being precipitated at the time, but only after standing. With Nylander's reagent there is only slight reduction. The urine is not fermented by yeast, it gives a strongly positive orcin reaction, and when heated with phenylhydrazine gives an osazone which melts in the crude state at about 150°C . In all the genuine cases so far described the urine has been optically inactive.

The present case, for which we are indebted to Dr C J Currie of Toronto who brought her to the notice of one of us, was, in all the above respects, like those previously described. It was only in an attempt to estimate the pentose by Neuberg's method¹ that any abnormality was met with. In only one instance, to our knowledge, has the pentose been actually isolated from a case of pentosuria, and that was by Neuberg in 1900². Using twenty liters of urine, Neuberg was able to obtain about twenty grams of the pentose as the diphenylhydrazone. This on decomposition with formalde-

¹ Neuberg and Wohlgenuth *Zeitschr f physiol Chem*, **xxxv**, p 35

² Neuberg *Berichte*, **xxviii**, p 2243

hyde yielded the sugar itself, which was found to be inactive arabinose. In our case, on attempting to estimate the pentose by means of diphenylhydrazine no insoluble hydrazone was obtained. An attempt was therefore made to isolate the pentose following the process exactly as described by Neuberg, but was fruitless. It was also found impossible to separate any crystalline derivative other than the phenylosazone, proceeding by way of the precipitate produced in the urine by basic lead acetate and ammonia, which contains all the reducing substance. That the substance is a pentose is shown by analysis of the osazone, but we have been unable to obtain any evidence that it is arabinose. The other two pentoses which give an osazone melting above 160°C , are ribose and arabino-ketose. The latter so far as we are aware, has not been encountered up to the present time in the examination of animal tissues or fluids. The fact that *d*-ribose had been shown by Levene and Jacobs to be present in some of the nucleic acids, suggests that there is some possibility of the pentose in the present case being inactive ribose.

The clinical history of the case is as follows

Mrs H, aged 32, a Russian Jew. Married, two children living. One child died at five and one-half months of pneumonia, one of scalds.

Previous illness. At about four years of age had a swollen hand with discharge of pus from opening on the dorsum, finally some bone discharged and the opening healed, leaving a transverse scar. A similar condition about the knee healed with no discharge of bone, at the same time also there was a discharging sore near one ankle and this the patient says still discharges at times. One year ago a child two years of age fell into a tub of boiling water and died an agonizing death. Patient was much upset, became very nervous and went to Philadelphia to live. In the summer of 1910 she was admitted to the Jefferson Hospital with a swollen leg. There were places on the leg like boils but no pus was present. She was told her veins were inflamed. While in the hospital she was told there was sugar in the urine and that she had a mild form of diabetes. She returned to Toronto in the autumn of 1910 and sought advice regarding the glycosuria. She was not losing in weight, general appearance good, though she was of a nervous type and much worried and anxious about herself. At times she showed a tendency to magnify her symptoms and reported to her physicians many minor aches and pains. She complained of being always tired and at times weak, occasionally her hands were heavy to raise or move. She perspired freely in the axillae under examination. The general physical examination yielded nothing of importance. Pulse and temperature normal. No polyuria or thirst.

Examination of the urine A routine examination of the urine showed that some reducing substance was present. The typical latent period before reduction was obtained with Fehling's solution. Nylander's solution was only slightly reduced. The urine was not fermented by yeast. Orcin reaction (Bial-Salkowski method), strongly positive. With phenylhydrazine a crystalline osazone was readily obtained. It crystallised out on cooling and melted in the crude state at 148 to 150°. On recrystallization from 20 per cent alcohol the melting point rose to 161 to 163°, finally on crystallization from 10 per cent alcohol containing a little pyridine it was obtained in long glistening yellow needles melting constantly at 163 to 164° C.

ANALYSIS The osazone was dried *in vacuo* over P_2O_5 at 35° C.

(1) 0.1431 gram gave 21.1 cc moist nitrogen at 16° and 753.2 mm Hg = 17.08 per cent nitrogen

(2) 0.1548 gram gave 23.1 cc moist nitrogen at 16° and 744.5 mm Hg = 17.09 per cent nitrogen

Theory for $C_{12}H_{10}N_4O_3$ requires 17.07 per cent nitrogen

Estimation of the pentose A forty-eight-hour quantity of 2650 cc was collected. One hundred cubic centimeters of this gave 0.168 gram crude phenylosazone corresponding to 0.0769 gram pentose or 2.04 grams in the two days. On attempting to estimate the pentose by Neuberg's method³, using 1000 cc of urine concentrated to 60 cc under reduced pressure at 35° C, no insoluble diphenylhydrazone such as arabinose gives, was obtained. Since the method for the estimation of arabinose in urine was worked out by Neuberg using normal urine to which varying amounts of the pentose were added, and since Neuberg has suggested that the pentose is excreted in combination with urea, it was conceivable that such a ureide might not react with diphenylhydrazine in the same way as the free sugar. This might therefore account for the failure to obtain the diphenylhydrazone in the above experiment. An attempt to isolate the pentose as diphenylhydrazone was therefore made using the process adopted by Neuberg for this purpose³. A liter of urine was concentrated to 50 cc at 35° under reduced pressure then poured into 360 cc of hot 95 per cent alcohol

³ Neuberg *Berichte*, LVIII, p. 2243

Cooled, filtered from salts, the latter were then dried in the air, ground up with ether, dried and extracted in a Soxhlet apparatus for eighteen hours with 95 per cent alcohol. The extract was added to the main alcoholic solution and the whole evaporated to 40 cc under reduced pressure. This residue was poured into 100 cc of hot 96 per cent alcohol, cooled, filtered and the filtrate boiled with animal charcoal for a few minutes. The fluid was again filtered and concentrated at 35° C to 44 cc. Three cubic centimeters removed for estimation of the pentose by titration, showed that 0.63 gram was present assuming the pentose to be arabinose. The alcoholic solution was therefore heated with 1 gram of diphenylhydrazine dissolved in a few cubic centimeters of alcohol in a boiling water bath for an hour. No diphenylhydrazone separated on standing. (The diphenylhydrazine used was a freshly prepared specimen which easily gave the characteristic hydrazone when heated with arabinose from gum arabic.) A second attempt was made using 4,700 cc urine following the same procedure, but was likewise unsuccessful. Taking advantage of the fact that the pentose is precipitated by basic lead acetate and ammonia, an attempt was made to isolate the pentose in this way. One liter of urine was precipitated with lead acetate, filtered, and the filtrate precipitated with basic lead acetate and ammonia. This removed all the reducing substance. The precipitate was decomposed with hydrogen sulphide and the solution so obtained concentrated to about 100 cc under reduced pressure at 35° C. It was neutralized to Congo red with sodium hydroxide, then to litmus with barium hydroxide, evaporated to a syrup *in vacuo* and poured into 200 cc of hot 95 per cent alcohol. The precipitated salts were filtered off after standing and the filtrate evaporated to small bulk under reduced pressure. This solution reduced very strongly and gave the characteristic osazone with phenylhydrazine but on treatment with diphenylhydrazine *p*-brom-phenylhydrazine or *p*-nitro-phenylhydrazine gave no crystalline hydrazone.

Since some authors have reported instances of more than one case of pentosuria in a family, the urines of the available blood relations have been tested. They were the son, daughter and brother, but all proved negative with Fehling's solution, the orcin reaction and phenylhydrazine.

NOTE Since sending the above results for publication we have been able, through the kindness of Dr P A Levene, who furnished us with a specimen of *d*-ribose, to compare the properties of the phenylosazone of this substance with those of the phenylosazone of the urinary pentose. A specimen of the osazone of *l*-arabinose (Merck) was also prepared for comparison. The osazones of the two active pentoses, which on theoretical grounds should have identical properties, melted at 162.3° , crystallized in the same manner and appeared to possess the same solubility in 10 per cent alcohol. The urinary pentosazone appeared to be less soluble in 10 per cent alcohol, and crystallized much better than the osazones from either of the active pentoses. No appreciable alteration in the melting point was obtained by mixing the urinary pentosazone with the osazones of the two active pentoses.

A BRIEF INVESTIGATION ON THE ESTIMATION OF LECITHIN

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Methods of determining lecithin in animal tissues are somewhat unsatisfactory, due to the lack of a routine method whereby lecithin itself can be separated and quantitatively estimated. By present methods it is determined along with related phosphatides, and the quantity reckoned from a phosphorus estimation by multiplication by a convenient factor.

The brief investigation, the results of which follow, is a comparison of several methods with a view to gaining evidence as to their accuracy.

In this laboratory, lecithin, in the strict sense, is estimated by an extraction with anhydrous alcohol and ether, evaporating the solvents and drying the resulting extracts, taking up with anhydrous ether, filtering and determining the phosphorus in the ethereal solution.

W Koch¹ states that owing to the difficulty of working under anhydrous conditions, this method is open to objection and that his method of separation of the lipoids with acid-chloroform-water is on this account preferable. This latter method is long and laborious when used in a routine way, and involves an undesirable correction for non-lipoid phosphorus clinging to the precipitate. The other method is more easily workable and its accuracy in so far as any method for lecithin is accurate is questioned only on the ground of the practicability of working under anhydrous conditions. The following comparisons were made in an effort to learn whether or not it is practicable to work under essentially anhydrous conditions, and whether solvents, not absolutely water-free, appreciably affect the results.

¹ *Journ Amer Chem Soc*, **XXI**, Dec, 1909

Five to eight gram samples of brain and liver contained in alundum extraction capsules were used in this work. They were thoroughly dried in a vacuum according to the method of Schackell,² being previously mixed in the capsule with sand to facilitate drying.

Three sets of samples were treated as follows:

1. Extracted with anhydrous reagents, the dried extracts taken up with anhydrous ether and filtered.

2. Extracted with 95 per cent alcohol and U S P ether, the dried extracts taken up with anhydrous ether and filtered.

3. Extracted with 95 per cent alcohol and U S P ether, and the lipoids separated by acid-chloroform-water according to Koch.

After complete desiccation the capsules containing the samples, the latter well covered with lipid-free absorbent cotton, were divided into three sets as above, placed in small bottles or flasks and covered with alcohol, absolute and 95 per cent respectively. The bottles were loosely stoppered with glass stoppers, placed in a water bath and the alcohol boiled gently for two hours. In this way extraction takes place at the boiling point of the solvent. The alcohol was then decanted and the capsules and samples washed several times with alcohol of the appropriate strength. This extraction and washing was repeated four times, making eight hours boiling in all. The capsules were then dried at a low temperature in hydrogen and extracted in the usual way for 16 hours with ether, using anhydrous or U S P material as above indicated.

The samples were now removed from the capsules and pulverized in an agate mortar. After returning to the capsules they were again extracted for 16 hours with ether of purity previously designated.

The ether and alcohol fractions were then freed from the solvents by evaporation at a low temperature, and the two fractions combined.

The absolute alcohol used in this work was prepared in this laboratory and was practically anhydrous. The anhydrous ether was made by redistillation from sodium of the ordinary anhydrous ether.

² *Amer Journ of Physiol*, LV, June, 1909

The extracts from sets 1 and 2 were now dried at 50° C in hydrogen, taken up with anhydrous ether and filtered through asbestos. The ethereal solutions were transferred to 250 cc Kjeldahl flasks, the ether evaporated and the residue decomposed with nitric and sulphuric acids. The phosphorus was determined, either by preliminary precipitation with magnesia mixture, or directly with molybdate solution, taking care that in the latter case an excess of ammonium nitrate was used and the precipitate was digested from four to six hours at 60° C.

The extracts of set three were treated according to the method of Koch with acid-chloroform-water, lipid phosphorus being determined on the precipitate and the necessary correction made for non-lipoid phosphorus.

The filtrates were perfectly clear.

Percentages of lipid phosphorus Averages of three to five determinations

METHOD	BRAIN		LIVER	
	First series	Second series	First series	Second series
Set I Anhydrous reagents, filtered	0 176	0 215	0 110	0 092
Set II Crude reagents, filtered	0 202	0 225	0 112	0 112
Set III Crude reagents, acid-chloroform-water	0 201	0 223	0 118	0 110

In comparing these results it should be borne in mind that in any method for lecithin estimation, taking for granted completeness of extraction and accuracy of technique, the probable error lies in the direction of results that are too high, due to inclusion of inorganic or other forms of non-lipoid phosphorus. The lower results, other things being equal, are probably more nearly accurate.

Although the variations in the results by the three methods are not great, those obtained by acid-chloroform-water treatment are slightly but uniformly higher than those obtained with anhydrous reagents and are probably, by that amount, too high.

It would therefore seem, that taking ordinary precautions to secure anhydrous reagents, the straight extraction method is preferable to the acid-chloroform-water separation.

With the purpose of further shortening the method, it was thought that possibly where anhydrous reagents were used, the filtering of the extract with anhydrous ether was unnecessary. To test this point, two sets of determinations were made on brain, liver and muscle. All samples were extracted with anhydrous reagents by the method given above. The dried extracts of one set were then taken up with anhydrous ether and filtered. The other set of extracts were analyzed for phosphorus directly without filtering. The results are given in the table below.

Percentages of lipid phosphorus Averages of four determinations

	EXTRACTS TAKEN UP WITH ANHIDROUS ETHER AND FILTERED	EXTRACTS NOT FILTERED PHOSPHORUS RUN DIRECT
Brain	0.248	0.249
Liver	0.153	0.153
Muscle	0.047	0.044

These results indicate that taking up the extracts with anhydrous ether and filtering is an unnecessary step in the determination, since practically identical results were obtained in both cases with all three tissues.

The most satisfactory method found therefore and the one in use in this laboratory, is that in which the combined alcohol and ether extracts are analyzed for phosphorus without previous filtration with ether, provided the necessary precautions are taken to secure dry reagents.

Crude reagents seem to render soluble certain forms of phosphorus which are not successfully separated from the lipoids by the acid-chloroform-water treatment.

The author wishes to express his thanks to Dr. E. B. Forbes for making this investigation possible.

THE PURINES OF MUSCLE¹

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The vast amount of work done on the purine bases in mammalia has shown that the two purines commonly found in fresh glands are adenine and guanine, and that hypoxanthine and xanthine, previously reported as having been found in various organs, occur only as the products of enzymatic changes of adenine and guanine, and are not found in the fresh glands² Muscle tissue, however, even when perfectly fresh, always shows the presence of more hypoxanthine than of any other purine, and the question therefore arose as to where this hypoxanthine came from Schittenhelm³ maintained that it was probably due to the conversion of adenine from nucleic acid by the ferment adenase, while Jones⁴ said that the hypoxanthine was formed in the muscle itself This work was therefore started in the hope of obtaining more light concerning muscle purines

GUANINE AND ADENINE

The fact that guanine occurs in muscle tissue in small amounts has long been known Adenine, however, is usually considered to be absent,⁵ though Mendel and Leavenworth⁶ showed that large amounts are obtainable from embryo calf muscle

¹ Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of California

² Oppenheimer's *Handbuch der Biochemie*, 1, p 610, 1908

³ Schittenhelm *Zeitschr f physiol Chem*, lxxii, p 248, 1909

⁴ Miller and Jones *Ibid*, lvi, p 393, 1909, Rohdé and Jones *this Journal*, vii, p 237, 1909-10

⁵ Cf Fränkel *Descriptive Biochemie*, p 124

⁶ Mendel and Leavenworth *Amer Journ of Physiol*, xxv, p 99, 1908

In this work it has been found that fresh rabbit muscle, after extraction with water at 65 to 70°C, upon digestion with dilute sulphuric acid yields guanine but not adenine. If the extraction be made with cold water, the residue after digestion yields both guanine and adenine and further it has been shown that this difference is due to the extraction of adenine from the muscle by the hot water. Three typical experiments are cited below in support of these statements.

1 A white female rabbit was etherized, then bled to death, and after skinning and cleaning, was immediately placed for fifteen minutes in distilled water at 60°C. The muscle was then stripped from the bones and ground three times. Four hundred and sixty grams of this hashed muscle, mixed with 700 cc of distilled water, were gradually heated to 60°C, then filtered through cloth, the residue being squeezed as dry as possible, and ground again. This process was repeated twelve times, using 600 cc of distilled water each time, and heating each extraction slowly to 60°C before filtering. The final residue, the moist weight of which was 371 grams, was digested with 1500 cc of 3 per cent sulphuric acid at 100°C for twenty-four hours. On treatment of the fluid with copper sulphate and sodium bisulphite in the usual way, 31 mgs of guanine were obtained. No trace of adenine was found, although a little of some other purine was present, probably hypoxanthine. The guanine obtained was purified through its silver salt and gave characteristic reaction when evaporated with strong nitric acid and treated with sodium hydroxide.

2 A white female rabbit was killed by bleeding after etherization, and its muscles removed and hashed. Five hundred and sixty-four grams of the hashed muscle were extracted with equal weight of distilled water for ten to fifteen minutes and the extract filtered through cloth. This process was repeated four times. The residue remaining was then digested for twenty-four hours with five times its weight of 3 per cent sulphuric acid. It yielded 65 mgs of guanine and 143 mgs of adenine picrate, equivalent to 53 mgs of adenine. There was also a little hypoxanthine(?) present.

3 A large black male rabbit was killed by bleeding after etherizing, and its muscles hashed. Nine hundred and forty-one grams of the hashed muscle were extracted five times with its own weight of distilled water. It was then extracted twice with like amounts of water, heated gradually to 55 to 57°C. The combined warm water extracts were acidified with 45 cc of concentrated sulphuric acid and digested for 24 hours at 100°C. It yielded 27 mgs of guanine and 62+ mgs of adenine picrate, equivalent to 25+ mgs of adenine.

Although adenine is found in the vegetable kingdom as in tea leaves in a free condition, it has almost never been found free in the tissues of higher animals, except in such abnormal tissues as cancers. Usually it is present in animal tissues as a constituent

of thymus nucleic acid. It would seem probable therefore that in the muscle also, adenine is present as a part of thymus nucleic acid. Only a portion of the guanine however, could thus be united since some guanine was always found in the tissue after the extraction by warm water had removed all of the adenine-containing molecules. All of the figures obtained in this work indicate that there is more guanine in muscle tissue than adenine. The following data are taken from Mendel and Leavenworth. From thirty embryos were obtained

Guanine hydrochloride	0.137 gram	= 0.093 gram guanine
Adenine picrate	0.185 gram	= 0.065 gram adenine
Hypoxanthine nitrate	0.057 gram	= 0.029 gram hypoxanthine

It may be partly due to this fact and also to the fact that guanine more readily crystallizes out of its solutions that the presence of guanine in muscles is better established than that of adenine.

Since striated muscle contains, in addition to the striped muscle, fibers, connective tissue and blood vessels, with their smooth muscle fibers, the following data obtained by Sieber and Dzierzowski⁷ on the purine content of the lungs are of interest, for in spite of the fact that the connective tissue of the lungs is not identical with that of muscle, it is convenient to look upon lung tissue as approximately equivalent to muscle tissue minus its striped muscle fibers. These workers found by Kossel's method that for every 100 grams of lung tissue, they obtained 60 mgs of xanthine, 164.7 mgs of guanine, 126.9 mgs of adenine, 179.6 mgs of hypoxanthine. Varying yields however were obtained with different methods. The high percentage of hypoxanthine present may possibly be due to the smooth muscle fibers of the blood vessels, etc., for Saito⁸ found that the purines of smooth muscle, as is the case with striated muscle, included hypoxanthine in preponderating amounts. To determine directly whether connective tissue contains adenine or not, I have digested connective tissue obtained from ox-tails and from the tendon of Achilles of the ox with sulphuric acid, 135 grams of connective tissue from the tendon of Achilles, digested with 675 cc of 3 per cent sulphuric acid, yielded 10.5 mgs of guanine, 25.5 mgs of adenine picrate, equivalent to about 10 mgs of adenine. It seems certain, therefore

⁷ Sieber and Dzierzowski *Zeitschr f physiol Chem*, lxi, p 259, 1909

⁸ Saito *This Journal*, iv, p 483, 1903

that some of the adenine and guanine in muscle must come from the connective tissue in it. It is possible that the large amounts of adenine and guanine obtained by Mendel and Leavenworth⁹ in embryo pig muscle, and also the high percentage of guanine found by Kossel¹⁰ in embryonic calf muscle, may really be due to the very high percentage of connective tissue in these embryonic muscles rather than to any specific feature of the muscles themselves. It is interesting to note that Saiki¹¹ found a trace of adenine in extract of smooth muscle—a substance not usually noted in extracts of striated muscle—and immediately afterward points out that smooth muscle contained very much more connective tissue than does striated muscle.

In order to further identify the guanine obtained from the various experiments, the picrate was made. Wulff¹² stated that guanine picrate begins to decompose gradually at 190°C, but the guanine picrate I obtained decomposed at nearer 260°C. Guanine was then prepared from the fresh thymus gland of the calf, by digesting it with 3 per cent sulphuric acid by volume, precipitating with copper sulphate and sodium bisulphite. The solution obtained by breaking down the copper purine was concentrated and the guanine precipitated from the hot solution by making it fairly strongly ammoniacal. After filtering off the mother-liquor the guanine was dissolved in hot dilute sulphuric acid and again precipitated while hot with ammonia in excess, filtered, and again this process was repeated. It was then changed into the silver salt and washed with ammonia, converted into the hydrochloride and evaporated carefully over a water-bath until just dry. This hydrochloride was then dissolved in hot water and filtered through hardened paper, and the picrate made by adding to it a solution of picric acid. This picrate also decomposed gradually from about 255 to 260°C. The following table compares the behavior of adenine and guanine picrates on heating.

<i>Adenine picrate</i>	<i>Guanine picrate</i>
At 240°C a trifle more brown than at first, and kept growing darker very slowly indeed	At 200°C began to grow slightly more brown
At 275°C still brownish yellow	At 212°C much more brown
At 280° to 281°C decomposed by turning rapidly black and melting	At 235°C orange
	At 240°C chocolate
	At 252°C brown
	At 258° to 260 C turned slowly quite black and melted

⁹ Mendel and Leavenworth *Loc cit*

¹⁰ Kossel *Zeitschr f physiol Chem*, viii, p 404, 1883-4

¹¹ Saiki *Loc cit*

¹² Wulff *Zeitschr f physiol Chem*, xvi, p 468, 1893

In this work it was found more convenient to make the picrates of adenine, guanine or hypoxanthine by adding a saturated solution of picric acid to the acid salt of the purine rather than the sodium picrate so often advised. A slight excess of the mineral acid did not interfere in any way with the reaction and it was found much easier to distinguish the purine picrates from the crystals of picric acid than from the needle-like crystals of sodium picrate in those cases in which for some reason or other the solution had to be very strongly concentrated. When picric acid is added to even a strong solution of guanine hydrochloride, no clouding is usually observed but in a very short time the extremely insoluble guanine picrate separates out as well defined crystals which soon sink to the bottom of the solution. When picric acid is added to adenine hydrochloride or sulphate, there is an immediate clouding of the solution due to the formation of very fine crystals of the adenine picrate, and these crystals do not settle out at all readily. The difference therefore between the two is well marked, although both picrates when once formed are extremely insoluble.¹² When dried at 105°C the guanine picrate always appears as sparkling crystals which do not readily give up their water of crystallization even when heated above 110°C,¹⁴ while adenine picrate, dried, shows no brilliancy and apparently readily gives up all its water at a little over 100°C.¹⁵ Guanine picrate seems to crystallize in several forms. The tree- or fern-like form, described so well by Wulff,¹⁶ has a distinctly redder appearance than adenine picrate, but those in the forms of long needles or platelets have the same color as adenine picrate. The redder, tree-like crystals were carefully picked out with a fine pair of forceps and placed in another receptacle, and when recrystallized from a little distilled water, came out as the lighter-colored platelets. The melting point determinations were all made on the yellower type. Usually guanine picrate dissolves very slowly in ammonia while adenine picrate dissolves almost immediately. Hypoxanthine also forms picrates of two forms—it sometimes appears as silky threads but more often as short, thick, six-sided crystals.¹⁷ As its solubility, however, is very much greater than either that of adenine or guanine, there is very little danger of contaminating the picrates of the two latter with hypoxanthine.

HYPOXANTHINE

By the digestion of fresh meat with dilute sulphuric acid we find that hypoxanthine is a normal constituent of muscle, but obviously we cannot thus learn anything concerning the condition of this hypoxanthine while in the living tissue. Rohdé and Jones¹⁸

¹² Bruhns *Zeuschr f physiol Chem*, **xiv**, p 533, 1890. Wulff *loc cit*

¹⁴ Wulff *Loc cit*

¹⁵ Bruhns *Loc cit*

¹⁶ Wulff *Loc cit*

¹⁷ Wulff *Loc cit*

¹⁸ Rohdé and Jones *Loc cit*

stated that hypoxanthine is probably formed in the muscle itself without the action of adenase, while Scaffidi,¹⁹ Mendel and Leavenworth,²⁰ Kennaway,²¹ and Krukenberg²² speak of "free purines" or "free hypoxanthine" of muscle. Most of the investigators using the term "free purines" seem to mean by that any purine not directly connected with a protein, or with a coagulable protein (see Scaffidi), but we know that there are several substances in commercial meat extract, such as inosinic acid, carnine, inosin, etc., which are neither compound proteins nor in any sense free hypoxanthine, although easily yielding the latter on decomposition. No reference was yet found which really considered carefully whether the so-called "free hypoxanthine" found in the muscle was free or not, for it is obvious that a method like that of Kruger-Schmid²³ as ordinarily carried out cannot be relied upon for such determinations. The first problem was therefore to see how much inosinic acid was present in fresh meat. The principal methods given for the preparation of inosinic acid are briefly as follows:

LIEBIG'S METHOD ²⁴ The cold extract of fresh meat was boiled to coagulate the proteins, and then the filtrate was evaporated to a very small volume at a low temperature to crystallize out the inosinic acid.

HAISER'S METHOD ²⁵ The commercial meat extract was boiled with absolute alcohol and the inosinic acid in the insoluble residue was precipitated as the silver salt after the elimination of all the phosphates with barium hydroxide. Levene's modification²⁶ of this consisted in extracting with 95 per cent alcohol instead of absolute.

BAUER'S METHOD ²⁷ The water solution of commercial meat extract, after clearing with animal charcoal, was freed from phosphates with barium acetate and hydroxide, and the inosinic acid was precipitated in an alkaline solution with basic lead acetate.

¹⁹ Scaffidi *Biochem Zeitschr*, **11**, p. 247, 1911.

²⁰ Mendel and Leavenworth *Loc cit*.

²¹ Kennaway *Biochemical Journal*, **v**, p. 188, 1910.

²² Krukenberg *Untersuchungen aus dem physiologischen Institut der Universität Heidelberg*, **III**, p. 217, 1880 (ref. from Mendel and Leavenworth).

²³ Hoppe-Seyler-Tierfelder, *Handbuch d. physiol.-chem. Analyse*, **VI**, p. 435 (ref. from Frankel's *Descriptive Biochem.*)

²⁴ Liebig *Ann d. Chem u. Pharm*, **111**, p. 257, 1847.

²⁵ Hauser *Monatsh f. Chem*, **16**, p. 190, 1895.

²⁶ Levene and Jacobs *Ber d. deutsch. chem. Gesellsch.*, **31**, p. 2704, 1908.

²⁷ Bauer *Berlin z. chem. Physik*, **1**, p. 345, 1907.

HAISER AND WENZEL'S METHOD ²⁹ The commercial meat extract, after freeing from phosphates, was neutralized and the inosinic acid precipitated with basic lead acetate

All but the first of these methods start with the commercial meat extract and not with the fresh meat, and the only one which starts with the meat itself has been found to yield uncertain results ²⁹ The following method in which boiling, long evaporations, and acidity are avoided, was therefore adopted in this work

A rabbit was etherized and killed by bleeding, and its muscles were separated as quickly as possible. The meat was then ground twice, and extracted with its own weight of cold, distilled water five times, allowing the meat to soak ten or fifteen minutes for each extraction. The meat was filtered through cloth, and squeezed as dry as possible each time, and the same cloth was used for all the filtrations. When the extractions are made with hot water, gelatin and other undesirable substances are also extracted which are difficult to separate from the inosinic acid. The united filtrate was then heated to 65°C to coagulate some of the proteins and filtered through paper. To the clear filtrate a saturated barium hydroxide solution was added to precipitate the phosphates and sulphates, and also most of the remaining proteins. The precipitation of proteins by barium hydroxide has already been used by Peters³⁰ in his preparation of thymus nucleic acid and has been found to be an extremely convenient reagent in this work. When the further addition of the barium hydroxide caused no more precipitation, the solution was warmed to 45 or 50°C with constant stirring, to make the precipitate form a coagulum which was easily filtered off. The filtrate was then exactly neutralized with dilute acetic acid and basic lead acetate immediately added to precipitate the inosinic acid, until all precipitation just ceased. An excess of basic lead acetate is to be avoided as it dissolves the precipitate. After the precipitate had settled the liquid was decanted into a filter and the precipitate washed once or twice by decantation with distilled water, pouring all the water through the filter. Finally, all the precipitate was also carefully washed into the filter. Then the paper with the lead precipitate in it, was placed in a beaker containing a little water and was beaten into a pulp, special care being taken that no large lumps of the lead precipitate remained. After slightly warming this mixture, some clear saturated solution of barium sulphide was added drop by drop while constantly stirring until the solution just began persistently to tarnish a well-cleaned silver coin when a drop of the solution was left on the coin one minute. A large excess of barium sulphide is to be avoided as the barium sulphide constantly but slowly changes to barium carbonate

²⁹ Hauser and Wenzel *Monatsh f Chem*, xxix p 157, 1908

²⁹ See Bauer, *Loc cit*

³⁰ Peters *This Journal*, v, p 373, 1911

when exposed to the air, and this precipitate is therefore sure to contaminate the final product. The solution was then warmed to 60°C and filtered warm. The filtrate was usually perfectly clear and apparently contained very little besides barium inosinate. After thoroughly washing the precipitate with warm water, the united filtrate was placed in a beaker with perpendicular sides and mixed thoroughly with five times its own volume of 95 per cent alcohol, and then left covered for twenty-four hours. In this way the barium inosinate was precipitated quantitatively at the bottom of the beaker. All the liquid that could be safely decanted was very carefully decanted off. When this operation was well done, it saved a great deal of time as the subsequent filtration was always very slow. The remaining liquid and the precipitate were then placed into a Buchner funnel provided with filter paper, and suction was applied. All the precipitate that passed through the filter, and some was sure to go through at first, was again placed on the filter until a perfectly clear filtrate was obtained. This filtration was always extremely slow, but fortunately did not require much of the operator's attention. It was finally washed with absolute alcohol and ether and allowed to dry at room temperature. The dry material was then separated as much as possible from the funnel and filter paper and the two last were carefully washed with a little hot water to dissolve out all the barium inosinate adhering to them. The major portion of the precipitate was then transferred to the same water and after carefully breaking up all lumps, was placed over a steam bath and constantly stirred. As soon as most of the material had dissolved, it was filtered hot through a small filter-paper. Should the material not readily dissolve in the amount of water present, a little more may be added, but a great excess should not be added. All the soluble products should easily dissolve without heating over 80°C. After filtering, the filter paper was washed free of the precipitate on it with a little hot water and this precipitate again digested over steam a short time and again filtered hot. The warm filtrate was allowed to cool slowly and was finally placed in the ice-chest. After twenty-four hours or more, the crystals of barium inosinate were filtered off and allowed to dry at room temperature. If the volume of mother-liquor was not too small, it was found advisable to concentrate it strongly at 50°C and then again leave in the ice-chest for a second crystallization.

Hauser³¹ in his method warns us of the danger of adding an excess of barium hydroxide and states that all the inosinic acid may be lost as an insoluble basic barium inosinate by doing so. In the article by Hauser and Wenzel³² this statement is very much modified, but from the following experiment it seems evident that the caution is wholly needless. Barium inosinate was dissolved in a little hot water and barium hydroxide, a saturated solution, was added

³¹ Hauser *Loc cit*

³² Hauser and Wenzel *Loc cit*

in gradually increasing amounts. No precipitate, however, formed either in the cold or on heating moderately. Then a clear solution of saturated hydroxide was taken and some pure dry barium inosinate added to it and very gently heated. All the salt dissolved in the solution. The solution was then carefully filtered through hardened paper, just neutralized with acetic acid and treated with a solution of silver nitrate. The characteristic silver inosinate precipitate formed, which immediately dissolved on adding ammonia. Of course, when the carbon dioxide of the air was allowed to act on the solution of barium inosinate in barium hydroxide, some barium carbonate was formed, but that was the only precipitate present even after standing several hours. It seems evident therefore that no loss of inosinic acid need be feared from an excess of barium hydroxide.

In attempting to get a good method for the preparation of inosinic acid various reagents were tried. The silver, copper and mercury salts were found unsuitable.

In concentrated solution of barium inosinate, neutral lead acetate gives a precipitate, but not when dilute, while basic lead acetate precipitates even from very dilute solutions. One milligram of barium inosinate, dissolved in 15 cc. of water gave a characteristic precipitate after ten or fifteen minutes. A great excess of either neutral or basic lead acetate completely dissolves the precipitate. The basic lead salt is insoluble in cold water and practically insoluble in hot. It settles rather quickly and so can be easily washed by decantation. It filters better than the silver salt and is not affected by light. An excess of carbon dioxide, however, should not be present.

Basic lead acetate and ammonia is perhaps a still better precipitating agent for inosinic acid but as this reagent precipitates also all the purine bases, gelatin, creatine, etc., which would ordinarily not be precipitated by basic lead acetate alone, the use of ammonia or any other alkali is to be avoided when the purity of the inosinic acid is a consideration.

In the purification of barium inosinate when the substance does not readily purify by repeated recrystallization with hot water, the crystals were dissolved in cold, very dilute, sodium hydroxide (about 0.3 per cent), after completely freeing the alkali from all carbonates with barium hydroxide. In this alkaline solution the barium inosinate dissolves rather readily, and

may be recovered again after filtration by almost neutralizing the solution with dilute acetic acid. As the barium inosinate is naturally extremely faintly alkaline to litmus, the yield is larger if the neutralization is not quite completed.

It is interesting to note that Hauser³³ states that he obtained from inosinic acid a substance agreeing in nearly all particulars with Steudel's hypoxanthine, but differing from his description in that it was precipitated with basic lead acetate. In this Weidel³⁴ seems to agree with Hauser, and cites the fact that Stadeler³⁵ precipitated part of his xanthine with basic lead acetate. On the other hand Rohd  and Jones³⁶ recommended the use of this basic acetate to clear the solutions of substances other than purines, and the same reagent was used in a method also previously followed by Neubauer³⁷ and others for the same purpose. Later even Hauser³⁸ used the same reagent to separate the inosinic acid fraction from the carnine fraction without, however, withdrawing or explaining his first statement so far as I know. It seemed wise, therefore, to obtain a better knowledge concerning this question. Hypoxanthine was therefore prepared from the commercial Liebig's meat extract by precipitating it as the copper salt, and then as the silver salt which was next dissolved in boiling nitric acid according to Neubauer's method. The silver nitrate salt of hypoxanthine which separated out was digested in weak ammonia, then broken down with hot dilute hydrochloric acid and the acid then evaporated away on a water bath. The salt was then dissolved in water and carefully precipitated as the free base by just neutralizing the solution with sodium hydroxide.

Ten milligrams of this dry base was carefully dissolved in 10 cc of distilled water by heating, forming a 0.1 per cent solution, and to the cooled solution basic lead acetate prepared exactly as directed in the U. S. Pharmacopeia, page 267, was added, but no precipitate formed. This, however, was a super-saturated solution from which crystals of hypoxanthine separated out when left

³³ Hauser *Loc cit*

³⁴ Weidel *Ann d Chem u Pharm*, clviii, p 353, 1871

³⁵ Stadeler *Ann d Chem u Pharm*, cxvi, p 102, 1860

³⁶ Rohd  and Jones *Loc cit*

³⁷ See Balke *Journ f prakt Chem*, clv (n s xlvii), p 552, 1893

³⁸ Hauser and Wenzel *Loc cit*

over night without the acetate Emil Fischer states that hypoxanthine is soluble in 65.5 parts of boiling water, in 1415 parts of water at 19°, in 1370 parts of water at 23°³⁹ Even when 10 mgs was dissolved in 5 cc of water, a 0.2 per cent solution, no precipitate formed with the lead salt, but when a 0.4 per cent solution of hypoxanthine was tested, the basic lead acetate did cause a precipitation The precipitate dissolved again when the solution was sufficiently diluted As the total purine content of the meat is generally thought to be about 0.2 per cent⁴⁰ it seems evident that when the weight of the water used for extraction equals the weight of the fresh meat to be extracted there is no danger of the basic lead acetate precipitating any of the free hypoxanthine This then gives a convenient method of separating hypoxanthine from inosinic acid—a method already used and published by Hauser and Wenzel⁴¹

Two attempts to determine quantitatively the amount of inosinic acid in rabbit meat were made with the process given above The first time 0.48 gram of the barium salt, $C_{10}H_{11}BaN_4PO_8 + 7\frac{1}{2} H_2O$, air dried, was obtained from 530 grams of meat, and the second time over 0.60 gram from 580 grams of meat This points strongly to the conclusion that a large part of the hypoxanthine must be in some other form besides that of inosinic acid if the total hypoxanthine content is anywhere near 0.2 per cent Indeed the fact that Balke⁴² by Neubauer's method in which he also used the filtrate from solutions treated with basic lead acetate, could get large amounts of hypoxanthine, proves the same contention

DISTRIBUTION OF INOSINIC ACID

Turning now to the distribution of inosinic acid in the different kinds of animals, we find that shortly after Liebig's⁴³ discovery of inosinic acid in 1847, more or less work was done to determine if the muscles of all animals contained this acid or not In 1848

³⁹ Ref from Beilstein *Handbuch d. org. Chem., Ergänzungsband*, III, 1708

⁴⁰ Scaffidi *Loc. cit.*, Burian and Hall *Zeitschr. f. physiol. Chem.*, XXXVIII, p. 336, 1903

⁴¹ Hauser and Wenzel *Loc. cit.*

⁴² Balke *Loc. cit.*

⁴³ Liebig *Loc. cit.*

Gregory⁴⁴ published the statement that he was unable to find inosinic acid in ox-heart, in pigeons, and in codfish, although he obtained fairly large amounts from some other animals. A little later Schlossberger⁴⁵ was unable to find any in human flesh. Since that time a vast amount of work has been done on the structure of inosinic acid by Hauser, Levene, and others, but very little seems to have been done concerning its distribution, except for the work of Creite,⁴⁶ so that Bauer⁴⁷ in 1907 still quoted Gregory's statement concerning the absence of inosinic acid in certain animals.

As it appeared a rather significant fact if inosinic acid were really absent in certain animals while present in their near relatives, a test was undertaken on the pigeon, which was the only one of Gregory's animals easily available at the time. The method followed was in general that of Hauser and Wenzel⁴⁸ with the exception that meat, instead of meat extract, was used, and the washing of the lead salt was carried on considerably more, as this part of the work was done before the method given above for the preparation of inosinic acid, was devised.

Five pigeons were etherized and killed by bleeding, immediately cleaned, and the meat freed from bones was ground and extracted with water at 60°C several times. The united filtrate was treated with saturated barium hydrate until no more precipitate formed.

The barium precipitated all the phosphates and most of the proteins in the solution. The filtrate was made neutral with dilute acetic acid and then very faintly alkaline with ammonia. Basic lead acetate was added until complete precipitation took place, but an excess was carefully avoided. The precipitate was then washed by decantation in tall cylinders by changing the water every three or four hours for several days. The acid was now liberated with hydrogen sulphide in the cold, powdered barium carbonate was added, and the whole heated over steam. The filtrate was next evaporated to a very small volume at about 50°C.

The resulting crystals were recrystallized until long beautiful platelets over a millimeter in length were obtained, which were in every respect exactly like those obtained from Liebig's meat extract by Hauser's earlier method.

⁴⁴ Gregory *Ann d Chem u Pharm*, lxxv, p 100, 1848

⁴⁵ Schlossberger *Ann d Chem u Pharm*, lxxvi, p 80, 1848

⁴⁶ *Zeitschr f rationelle Med*, xxxv, p 195

⁴⁷ Bauer *Loc cit*

⁴⁸ Hauser and Wenzel *Loc cit*

The yield was only about one-tenth of a gram and responded to all the usual tests for the barium salt of inosinic acid

This method probably does not give even approximately quantitative results, but it leaves no doubt that inosinic acid is present in the pigeon, and all statements concerning the absence of this acid in birds or mammals, should, I think, be held in considerable doubt until some better and more quantitative methods be used

Attempts were made to see if the smooth muscle tissue also contained inosinic acid As Salki⁴⁹ had already pointed out that hypoxanthine constitutes there also the largest portion of the purine bases, the presence of inosinic acid was naturally expected For this purpose bladders from oxen just killed were taken, carefully trimmed from loose connective tissue, red muscle fibers of the neck, fat, etc, cut open and washed, then ground and treated exactly as was the rabbit meat It was found however that on heating the cold water extract the coagulum would not separate from the mother liquor as in the case of striated muscle, but formed a fine milky precipitate which almost prevented filtration This was found to be due to the lack of acidity of the extract of smooth muscle, for as shown by Halliburton and others,⁵⁰ the extract of striated muscle is always slightly acid, while that of smooth muscle⁵¹ is not A few drops of weak acetic acid were therefore added and the solution vigorously stirred, which made the subsequent filtration very much easier On adding basic lead acetate until precipitation completely ceased, it was found that the solution always contained a fine suspension of some lead precipitate which would not settle even after hours of standing As the basic lead precipitate of inosinic acid settles almost completely under similar circumstances, this milky solution was decanted off The heavier precipitate was treated with fresh water and the solution again decanted off after all the heavier precipitate had settled This was repeated until most of the fine precipitate had been washed away The subsequent treatment of the lead precipitate was conducted exactly as in the case of rabbit meat No inosinic acid could be identified from the ox bladders, although the attempt

⁴⁹ Salki *This Journal*, iv p 483, 1908

⁵⁰ Halliburton *Journ of Physiol*, viii, p 133, 1887

⁵¹ Vincent *Zeitschr f physiol Chem*, xxxiv p 417, 1901-2

was made three times, using respectively 560 grams, 810 grams, and 2660 grams of the fresh bladder meat for the trials. Some crystalline substance was indeed obtained but it did not give the inosinic acid tests tried above. Whether there is any inosinic acid in smooth muscle or not, is therefore an open question but it seems evident that the amount must be much less than in the striated muscle fibers.

CONCLUSION

From the facts previously stated, we conclude that muscle tissue contains small amounts of adenine and guanine, very likely in the form of thymus nucleic acid, and that some of this, perhaps all of it, is found in tissues other than the striated muscle fibers themselves. More guanine, however, is present than adenine.

The inosinic acid of striated muscle fibers represents only a fraction of the total hypoxanthine present, but we are not at all certain whether the remaining hypoxanthine is free, that is, uncombined with any complex organic radicle, or not.

Inosinic acid is probably present in the striated muscles of all warm blooded animals, although in apparently very varying amount. It may be absent in smooth muscle tissue.

Since the inosinic acid can be so easily obtained by extraction with cold water, it seems hardly probable that it is confined to the nuclei of the muscle cells. This supposition is still more strengthened by the fact that the nucleic acid in other tissues are found, after death at least, united with proteins in the form of nucleoproteins, which are rather insoluble in cold water and which must be first digested with some alkali or acid to liberate the nucleic acid. This is well shown by the method of Steudel and Brigl⁵² for the preparation of guanylic acid, also by Peter's method for the preparation of thymus nucleic acid. Inosinic acid, however, is obtained in fairly pure condition by precipitating the cold water extract of meat with basic lead acetate. Inosinic acid therefore is probably a nucleic acid only from the chemical standpoint and not from the histological.

⁵² Steudel and Brigl *Zeitschr f physiol Chem*, lxxiii, p 40, 1910

THE INFLUENCE OF COCAINE UPON METABOLISM WITH SPECIAL REFERENCE TO THE ELIM- INATION OF LACTIC ACID

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The introduction of cocaine into the organism is followed by such well defined symptoms that an almost specific influence upon the nervous system is indicated. In the main, it is to this aspect of its action upon the body that the very extensive literature¹ regarding this drug relates. Definite knowledge of the effect of cocaine upon general metabolism is meagre although the picture presented by the cocaine habitué is sufficiently characteristic to lead one to infer that ultimately at least the nutritional rhythm must be altered. The widespread employment of cocaine as an ingredient of various types of proprietary remedies and the large number of cases of cocaineism makes pertinent at this time an inquiry into the influence upon metabolism of the drug under discussion.

The observation of Araki² that lactic acid appears in the urine in unusually large quantities after cocaine injections considered in connection with the findings of Wallace and Diamond³ that cocaine causes vacuolization of the liver cells of rabbits suggested the possibility of a disturbance in intermediary metabolism. In the present paper the relation of cocaine poisoning to lactic acid output is shown and the influence of the nutritive condition of the animal upon this type of acidosis is discussed. It is also demonstrated that in spite of the marked symptoms characteristic of

¹ Cf. Richet *Dictionnaire de physiologie*, iv, p. 1, 1900.

² Araki *Zeitschr f. physiol. Chem.*, vi, p. 335, 1891.

³ Reported at the 19th Annual Meeting of the American Physiological Society, New York, 1907.

chronic cocaine poisoning general metabolism is only slightly changed from the normal even though the quantity of drug administered is sufficient to finally cause death. These observations serve as a further illustration of the tenacity with which the organism adheres to the fundamental laws underlying its metabolic processes, in other words, another example of the "factor of safety" principle is encountered in cocaine poisoning.

THE INFLUENCE OF COCAINE UPON METABOLISM, AS INDICATED
BY ITS EFFECT UPON NITROGENOUS EQUILIBRIUM AND PROTEIN
AND FAT UTILIZATION

Methods The experiments were planned so that the animals (dog and rabbit) employed were kept upon a fixed diet and cocaine administered subcutaneously at a time sufficiently long after a meal to avoid the danger of food being vomited. During the first period of the experiments the drug was given once daily, later the animal was kept under the influence of cocaine the greater portion of each day by repetition of the injection.

Lactic acid was estimated by the Ryffel⁴ procedure. The Folin method as modified by Steel⁵ was employed in the determination of ammonia in the urine of rabbits. The other determinations were carried out according to the well known methods usually employed in this laboratory. Urine was collected in twenty-four hour periods by catheterization (dogs) or by pressure on the bladder through the body wall (rabbits). Unless otherwise noted all urines of dogs were acid in reaction to litmus. The rabbits' urines were alkaline throughout.

Description of experiments Experiments 1 and 2. In these observations dogs 50 and 51 were kept for several days previous to the investigation upon the diet arranged for the experimental trials in order to bring them as nearly as possible into a condition of nitrogenous equilibrium. A fore-period was followed by an interval during which the animals received daily subcutaneous injections of cocaine hydrochloride (Kahlbaum's crystalline product) dissolved in water. In addition to a constant diet throughout the experiment the animals received, also, a fixed water intake.

⁴ Ryffel *Journ of Physiol*, *xxxix*, p. v, 1909-10

⁵ Steel *This Journal*, *viii*, p. 365, 1910-11

Protocol of Experiment 1

Dog 50, weighing 12.8 kilos, was normal in every respect except that she was extremely deaf. The diet consisted of 200 grams meat, 80 grams cracker meal, 40 grams lard, 10 grams bone ash, and 300 cc water. The total nitrogen intake amounted to 7.40 grams nitrogen daily with sufficient fat and carbohydrate to yield approximately 70 calories fuel value per kilo of body weight. Each day food was given at 9.30 a.m. and the first cocaine injection at 3.30 p.m.

On *October 20* the cocaine period was begun. Just before the cocaine injection the rectal temperature was 38.6°C and two hours later had risen to 39.0°C . The pupils showed extreme dilatation.

October 21 In the morning the dog seemed normal and ate food with evident relish. Temperature before cocaine administration was 38.6°C and had risen to 40.0°C two hours later. About 45 minutes after the injection the animal exhibited peculiar movements of the head which were constant. The dog was extremely restless. The pupils were greatly dilated.

October 22 The dog was apparently normal at meal time. Symptoms after cocaine injection similar to those of previous days.

October 22 Symptoms unchanged.

October 24 Rectal temperature at 9.30 a.m. = 38.8°C , just before injection at 3.30 p.m. = 38.6°C , at 4.30 p.m. = 40.9°C .

At 4.30 p.m. the heart action was very slow but strong. Arrhythmic beating was in evidence. There was extreme dilatation of pupil. The animal was very much excited and the head was constantly moved up and down. Usually the animal was too deaf to pay attention to any sound, but at this time it would respond to a call.

October 25 In the morning the dog appeared normal and devoured food as usual.

Temperature at 9.30 a.m. = 38.8°C , just before injection, at 3.30 p.m. = 38.8°C , at 5.00 p.m. = 41.1°C .

The movements of animal were more pronounced and there was much more excitation after cocaine administration than had been observed at any previous time. The peculiar irregularity of the heart was again in evidence at 5.00 p.m. although previous to the injection, the beat was normal.

October 26 The appetite of animal was ravenous.

Temperature at 9.30 a.m. = 38.6°C , just before injection, at 3.30 p.m. = 38.6°C , at 4.00 p.m. = 41.6°C , at 5.00 p.m. = 40.9°C .

It was apparent that the animal had become much more sensitive to the cocaine since the usual daily injection was followed by greatly augmented symptoms of excitation. These lasted for a period of two hours.

October 27 The dog devoured food with apparent relish.

Temperature at 3.15, just before injection = 38.6°C , at 3.45 = 41.2°C , at 4.15 = 41.6°C , at 4.45 = 40.9°C , at 5.15 = 39.8°C .

The symptoms of excitation and pupil dilatation appeared within fifteen minutes after cocaine administration. Apparently the peculiar head movements were caused by an attempt to push the head out of the cage.

TABLE 1
Experiment I—Dog 50

Fore Period

(Daily Nitrogen Intake = 7.40 grams)

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE					FECES				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid	Weight		Water	Total Nitrogen	Ether Extract
								Moist	Dry			
1910	mgms	kilos	cc		gms	gms	mgms	gms	gms	per cent	gms	gms
October												
15		12.8	300	1.020	6.15	0.28 *(4.5)	49	26.8	11.6	56		
16		12.8	300	1.020	6.18	0.30 (4.8)	49	32.6	14.0	57		
17		12.8	310	1.021	6.03	0.27 (4.4)	48	19.0	12.5	34	3.30	6.09
18		12.8	300	1.020	6.24	0.29 (4.6)	49	29.0	15.0	49		
19		12.8	300	1.020	6.18	0.26 (4.2)	48	96.0	46.0	51		
Average per day		12.8	302	1.020	6.15	0.28 (4.5)	48	40.7	19.8	49	0.66	1.22

First Cocaine Period

20	128	12.8	410	1.020	6.45	0.26 (4.3)	54	51.0	26.0	49		
21	128	12.7	275	1.025	5.88	0.24 (4.0)	56	60.0	28.0	53		
22	128	12.6	260	1.020	6.15	0.34 (5.5)	60	29.0	15.0	49		
23	128	12.6	270	1.021	5.64	.28 (4.9)	64	35.0	17.0	51		
24	128	12.6	210	1.026	5.73	0.35 (6.1)	61	47.0	24.0	49		
25	128	12.5	240	1.030	6.75	0.34 (5.0)	54	35.0	20.0	42	8.07	17.83
26	128	12.4	170	1.040	6.66	0.30 (4.5)	53	51.0	30.0	41		

* Figures in brackets indicate percentages of total nitrogen

TABLE 1—Continued

First Cocaine Period—Continued

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE					FECES				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid	Weight		Water	Total Nitrogen	Ether Extract
								Molst	Dry			
1910	mgms	kilos	cc		gms	gms	mgms.	gms	gms	per cent	gms	gms
October												
27	128	12 3	165	1 045	6 12	0 27 (4 4)	70	40 0	24 0	40		
28	128	12 3	160	1 046	6 54	0 30 (4 5)	76	50 0	24 0	52		
29	128	12 2	170	1 040	6 12	0 33 (5 3)	71	47 0	25 0	47		
30	128	12 2	165	1 041	6 06	0 30 (4 9)	78	44 0	24 0	45		
Average per day	128	12 5	227	1 032	6 19	0 30 (4 8)	63	44 0	23 0	47	0 73	1 62

Second Cocaine Period

November												
1	256	12 2	170	1 040	8 25	0 36 (4 3)	84	46 0	21 0	54		
2	256	11 4	140	1 050	6 84	0 35 (5 1)	83	52 0	32 0	39		
3	256	11 3	120	1 052	5 94	0 31 (5 2)	79	53 0	33 0	38	3 55	13 95
4	256	11 2	125	1 050	6 18	0 31 (5 0)	80	50 0	23 0	54		
Average per day	256	11 5	138	1 048	6 80	0 33 (4 9)	81	50 0	27 0	46	0 88	3 48

*Balances**Fore Period*

	<i>grams</i>		<i>grams</i>
Nitrogen in food	37 00	Ether extract in food	323 20
Nitrogen in excreta		Ether extract in feces	6 09
Urine	30 78		
Feces	3 30	Fat utilized	317 11
		Fat utilization =	98 per cent
Nitrogen balance	+2 92		
Per day	+0 58		
Nitrogen Utilization =	91 per cent		

First Cocaine Period

	<i>grams</i>		<i>grams</i>
Nitrogen in food	81 40	Ether extract in food	711 04
Nitrogen in excreta		Ether extract in feces	17 83
Urine	68 10		
Feces	8 07	Fat utilized	693 21
		Fat utilization =	98 per cent
Nitrogen balance	+5 23		
Per day	+0 47		
Nitrogen utilization =	90 per cent		

Second Cocaine Period

	<i>grams</i>		<i>grams</i>
Nitrogen in food	29 60	Ether extract in food	258 56
Nitrogen in excreta		Ether extract in feces	13 95
Urine	27 21		
Feces	3 55	Fat utilized	244 61
		Fat utilization =	94 per cent
Nitrogen balance	- 1 16		
Per day	- 0 29		
Nitrogen utilization =	88 per cent		

toward the light During the remainder of this period which was concluded on October 31 no new features developed

It was planned to begin the second cocaine period on October 31 by giving two injections of the drug, at 12 00 m and 4 00 p m respectively The first injection caused vomiting which contaminated the urine This period was therefore, commenced on the next day, November 1 On this date cocaine in doses of 128 mgrms each was administered at 3 00 p m and 5 00 p m Just previous to the first injection the temperature was 38 5° C, at 5 p m, 40 0° C, at 6 00, p m, 40 9° C The dog was in a state of extreme activity during this time

November 2 Cocaine was injected as on November 1 The conditions of the animal had, however, undergone a marked change since all movements were executed in a weak and uncertain manner

TABLE 2

*Dog 51**Fore Period*

(Daily Intake of Nitrogen=4.72 grams)

DATE	DAILY DOSE OF COCAINE mgms	BODY WEIGHT kilos	URINE					FECES				
			Volume cc	Specific Gravity	Total Nitrogen gms	Ammonia Nitrogen gms	Lactic Acid mgms	Weight		Water per cent	Total Nitrogen gms	Ether Extract gms
								Molat	Dry			
1910												
November 30		8.3	120	1.040	4.56	0.21 *(4.6)	45					
December 1		8.2	175	1.035	4.25	0.23 (5.1)	46	52.0	23.0	56		
2		8.2	165	1.036	4.23	0.23 (5.1)	51	17.0	10.0	42		
3		8.2	165	1.036	4.24	0.17 (4.0)	51	29.0	18.0	38	2.10	5.46
4		8.2	125	1.040	4.21	0.16 (3.7)	45	20.0	11.0	45		
5		8.2	175	1.030	4.20	0.18 (4.2)	48	15.0	10.0	33		
Average per day		8.2	154	1.036	4.28	0.19 (4.4)	47	22.0	12.0	42	0.35	0.91

Cocaine Period

6	123	8.2	220	1.026	4.44	0.18 (4.0)	58	11.0	5.0	55		
7	123	7.6	200	1.030	3.84	0.14 (3.6)	73	23.0	11.0	51		
8	123	7.6	155	1.035	4.35	0.16 (3.6)	74					
9	123	7.6	150	1.034	4.29	0.17 (3.9)	70					
10	123	7.6	155	1.035	3.80	0.16 (4.2)	79	42.0	22.0	46		

* Figures in brackets indicate percentages of total nitrogen

TABLE 2—Continued
Cocaine Period—Continued

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE					FECES				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid	Weight		Water	Total Nitrogen	Ether Extract
								Moist	Dry			
1910	mgms	kilos	cc		gms	gms	mgms	gms	gms	per cent	gms	gms
December												
11	123	7.6	250	1.030	4.21	0.18 (4.2)	85				3.89	32.54
12	123	7.6	130	1.040	4.50	0.18 (4.0)	90	50.0	25.0	50		
13	123	7.5	140	1.035	2.88	0.16 (5.5)	79	11.0	6.0	45		
14	123	7.5	135	1.037	3.15	0.16 (5.0)	90	44.0	31.0	30		
15	123	7.5	145	1.033	4.56	0.19 (4.1)	79	20.0	12.0	40		
								49.0	25.0	49		
Average per day	123	7.6	168	1.033	4.00	0.17 (4.2)	78	25.0	13.7	45	0.39	3.25

Balances

Fore Period

Nitrogen in food	grams	28.32	Ether extract in food	grams	243.60
Nitrogen in excreta			Ether extract in feces		5.46
Urine	25.69				
Feces	2.10	27.79	Fat utilized		238.14
Nitrogen balance		+0.53	Fat utilization = 97 per cent		
Per day		+0.08			
Nitrogen utilization = 92 per cent					

Cocaine Period

Nitrogen in food	grams	47.20	Ether extract in food	grams	406.00
Nitrogen in excreta			Ether extract in feces		32.54
Urine	40.02				
Feces	3.89	43.91	Fat utilized		373.46
Nitrogen balance		+3.29	Fat utilization = 91 per cent		
Per day		+0.33			
Nitrogen utilization = 91 per cent					

November 3 The dog showed signs of diminished appetite Conditions remained unchanged

November 4 Conditions about as usual Animal appears weak

November 5 The dog died twenty-five minutes after the first cocaine injection Just before death the dog was in a state of extreme activity This was rapidly followed by a period of partial paralysis culminating in respiratory failure Further data concerning this experiment may be found in Table 1, pp 238-240

Protocol of Experiment 2 Dog 51

A fox terrier bitch of 8.3 kilos was placed upon a fixed diet composed of 125 grams meat, 60 grams cracker meal, 20 grams lard, 10 grams bone ash and 150 cc water for a period of 10 days previous to the actual fore period of the experiment The nitrogen content of this diet amounted to 4.72 grams, the fuel value was approximately 69 calories per kilo body weight

November 30 On this date the fore period of six days was begun

December 6 The cocaine period was commenced by the injection of 123 mgms cocaine at 3.00 p.m. No rise in temperature could be observed The only symptoms noticeable were salivation and pupil dilatation

December 7 About one-half hour after the administration of cocaine the dog became markedly excited, the bodily movements not being under perfect control Pupil dilatation was extreme and the arrhythmic heart beat was evident

Each day up to December 12 the symptoms of excitement etc were noticeable but unchanged in character

December 12 Shortly after the cocaine injection the animal became completely paralyzed in the hind-quarters The jaws and tongue were kept constantly in motion as though the animal was tasting something unpleasant The dog remained in this condition for several hours during which she appeared deaf and blind

December 13 The animal seemed normal although somewhat weak The weakness became more and more noticeable and on December 15 the experiment was terminated

For other data associated with this animal see Table 2, pp 241-242

DISCUSSION OF RESULTS

From the details of the protocols and tables submitted it is apparent that the most obvious symptoms arising from cocaine injections in the doses given are distinctly of nervous origin A significant influence is also exerted upon the heat regulating mechanism whereby the temperature is quite markedly increased for a short period after which there is a gradual return to the normal⁶ With daily doses of 10 mgms of cocaine hydrochloride

⁶ Reichert *Centralbl f d med Wissenschaften*, 1889, p 444

per kilo of body weight no appreciable influence can be detected upon the course of nitrogenous metabolism nor upon the utilization of protein and fat although body weight shows an appreciable decline

When injections of 15 mgms cocaine per kilo are daily administered fat utilization is very slightly impaired and is accompanied by a decreased body weight. Doses of 20 mgms per kilo per day divided into two injections show a fairly distinct detrimental influence upon both protein and fat utilization and for the first time a slight negative balance was in order. Body weight was markedly diminished under this dosage.

The water excretion of Dog 50 was quite distinctly diminished under cocaine when compared with that of the fore-period. This finding does not hold true for Dog 51. The difference may be explained perhaps by the fact that Dog 50 was apparently much more sensitive in its reaction to cocaine with respect to the temperature raising influence than was Dog 51. Assuming this to be true more water was probably eliminated by the lungs in the first case than in the second which would account for lessened water elimination by the kidney.

THE INFLUENCE OF COCAINE UPON THE ELIMINATION OF LACTIC ACID IN THE URINE

The presence of lactic acid in the urine in appreciable quantities has been a subject of much investigation and discussion resulting in a multiplicity of conflicting theories with respect to its significance. Out of the enormous literature⁷ relative to lactic acid only a few references that have a bearing upon the present paper may be cited.

Thus, Araki⁸ has demonstrated that lactic acid appears in the urine in the absence of a sufficient supply of oxygen induced by various types of toxic compounds and epileptic seizures. The older work of Spiro⁹ indicating that increased muscular activity leads to lactic acid excretion finds confirmation in the recent investiga-

⁷ Ryffel *Quarterly Journ of Med*, III, p 413, 1909-10

⁸ Araki *loc cit*

⁹ Spiro *Zeitschr f physiol Chem*, I, p 111, 1877

tions of Ryffel¹⁰ and Feldman and Hill¹¹ According to the latter authors the appearance of lactic acid in the urine may be greatly diminished by breathing oxygen before and after exertion They conclude that the increased production of lactic acid by the muscles is due to oxygen want, a view that was earlier denied by Ryffel¹²

Viewed from the standpoint of ultimate origin, it is possible that lactic acid is intimately associated with the carbohydrate store of the body, for Araki found, under the experimental conditions, less lactic acid in the urine of starving animals than could be demonstrated in the urine of those well fed On the other hand, phosphorus, which leads to a disappearance of the carbohydrate store, causes a large output of lactic acid which may be accompanied by an increased elimination of ammonia¹³ It is presumed that the increase of the latter urinary constituent is for the purpose of neutralizing the lactic acid produced

In the experiments to be recorded the rabbits were kept upon a diet consisting of 300 grams of carrots and 20 grams oats which experience had demonstrated would usually be entirely eaten each day

Experiment 3 Rabbit B

During each day of the fore period this animal left small portions of the carrots uneaten After the subcutaneous cocaine injections no food was ever left For the first two days of the cocaine period no evidences of abnormal symptoms were observed On the third day, however, there was considerable dilatation of the pupil Beginning with November 9, the tenth day of administration, irritability and restlessness were noticeable The appetite remained good, all food being eaten shortly after the daily cocaine administration About 10 minutes after cocaine injection on November 11 the animal was seized with convulsions and respiration almost ceased, but recovery was complete three-quarters of an hour later On the succeeding two days convulsions were in evidence shortly after cocaine administration, but in each instance recovery was complete The animal died in a convulsion on November 14 The liver which was immediately excised contained 8 per cent of glycogen

From the data in Table 3 it will be observed that the injections of cocaine were progressively increased from approximately 15 mgms per kilo to 20

¹⁰ Ryffel *Journ of Physiol*, xxix, p xiv, 1909

¹¹ Feldman and Hill *Journ of Physiol*, xli, p 439 1911

¹² Ryffel *Journ of Physiol*, xxix, p xiv, 1909

¹³ Mandel and Lusk *Amer Journ of Physiol*, xvi, p 129, 1906

TABLE 3
Rabbit B
Fore Period

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lact Acid
1910	mgms	kilos	cc		grams	mgms	mgms
October							
26		2 38	200	1 016	1 00	2 5 (0 25)*	9
27		2 38	110	1 025	0 76	1 8 (0 23)	8
28		2 34	120	1 024	0 75	1 8 (0 23)	10
29		2 32	105	1 025	0 93	1 8 (0 19)	10
30		2 32	125	1 025	0 96	1 4 (0 15)	12
Average per day		2 34	132	1 023	0 88	1 8 (0 21)	10

Cocaine Period

31	33	2 32	125	1 025	0 90	1 0 (0 11)	11
November							
1	34 5	2 32	160	1 021	0 97	1 08 (0 19)	11
2	34 5	2 32	190	1 020	0 93	2 7 (0 29)	13
3	34 5	2 32	215	1 018	0 80	2 7 (0 33)	10
4	34 5	2 32	215	1 019	0 74	2 3 (0 31)	13
5	34 5	2 32	250	1 015	0 71	2 7 (0 8)	12
6	34 5	2 32	210	1 016	0 73	1 8 (0 24)	14
7	46	2 30	185	1 018	0 67	1 8 (0 27)	12
8	57 6	2 30	210	1 016	0 59	1 8 (0 30)	15

TABLE 3—Continued
Cocaine Period—Continued

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid
1910	mgms	kilos	cc		grams	mgms	mgms
November 9	69	2 26	235	1 015	0 61	1 8 (0 30)	26
10	89	2 22	195	1 020	0 61	6 3 (1 0)	25
11	101	2 24	180	1 020	0 63	1 8 (0 28)	33
12	101	2 28	200	1 024	0 82	1 1 (0 13)	39
13	101	2 30	185	1 024	0 88	1 1 (0 12)	51
Average per day		2 29	196	1 018	0 75	2 2 (0 30)	20

* Figures in brackets indicate percentages of total nitrogen

mgms on November 7, to 25 mgms per kilo on November 8, to 30 mgms on November 9, to 40 mgms on November 10, and finally to 45 mgms per kilo on November 11. Frequent tests throughout the cocaine period failed to demonstrate an appreciable rise in rectal temperature.

Experiment 4 Rabbit C

This animal behaved in a manner very similar to Rabbit B. A rise in rectal temperature of about 0.5° C was the maximum increase shown during the period of observation. The daily dose of cocaine given varied from approximately 10 mgms per kilo on November 29 and 30, to 20 mgms on December 1 to 6 inclusive, and from this time to the end of the experiment the animal received approximately 34 mgms cocaine per kilo body weight.

From the data in Tables 3 and 4 with rabbits and those in Tables 1 and 2 with dogs, it is evident that cocaine causes an appreciable increase in the elimination of lactic acid in the urine. In a general way the quantity of lactic acid thus excreted is in direct proportion to the amount of cocaine injected. The output of ammonia, however, does not appear to be significantly increased by the augmented elimination of lactic acid, an indication that in

TABLE 4
Rabbit C
Fore Period

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid
	<i>mgms</i>	<i>kilos</i>	<i>cc</i>		<i>grams</i>	<i>mgms</i>	<i>mgms</i>
1910 November							
21		2 26	290	1 014	0 80	1 0 (0 12)*	24
22		2 24	295	1 014	0 85	1 0 (0 12)	20
23		2 20	245	1 015	0 83	3 6 (0 43)	23
24		2 18	235	1 016	0 83	4 5 (0 54)	22
25		2 20	230	1 016	0 80	3 6 (0 45)	22
26		2 22	190	1 018	0 82	3 6 (0 44)	20
27		2 24	200	1 019	0 81	4 5 (0 55)	21
28		2 26	230	1 018	0 83	4 5 (0 54)	23
Average per day		2 22	239	1 016	0 82	3 2 (0 39)	22

Cocaine Period

29	20	2 26	270	1 017	1 18	4 5 (0 38)	23
30	20	2 24	245	1 020	1 40	4 5 (0 32)	23
December 1	45	2 26	240	1 021	1 30	3 6 (0 27)	24
2	45	2 24	230	1 021	1 26	3 6 (0 27)	24
3	45	2 26	220	1 022	0 97	3 6 (0 37)	25
4	45	2 30	230	1 022	0 86	4 5 (0 52)	26

TABLE 4—Continued

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid
	mgms	kilos	c.c.		grams	mgms	mgms
1910 December							
5	45	2 30	215	1 022	0 93	4 5 (0 49)	30
6	45	2 30	215	1 022	0 80	5 4 (0 67)	33
7	75	2 26	205	1 022	0 74	6 3 (0 85)	33
8	75	2 24	225	1 020	0 72	8 1 (1 12)	36
9	75	2 26	190	1 024	0 83	9 0 (1 08)	40
10	75	2 30	230	1 020	0 95	9 0 (0 94)	42
Average per day		2 26	226	1 022	0 99	5 5 (0 55)	30

Figures in brackets indicate percentages of total nitrogen

this connection lactic acid may be neutralized by some base other than ammonia. This is particularly true for dogs, but does not hold quite so well with rabbits, for with Rabbit C the output of ammonia paralleled closely the elimination of lactic acid.

The influence of diet upon lactic acid elimination under the experimental conditions may be indirectly inferred from the data of Table 5 obtained from Dog 52 during a *period of inanition*. Here it will be observed that in spite of largely increased doses of cocaine lactic acid output fell considerably. The larger quantities of lactic acid excreted during the first few days of the experiment may perhaps be explained on the assumption that the carbohydrate store of the body during this interval had not been depleted. As soon as this condition had been reached a diminution in lactic acid output took place. These results are in harmony with the theories outlined by Araki, but are in opposition to the observations reported for pernicious vomiting of pregnancy where lactic acid is eliminated¹⁴ in the urine probably as a result of the inanition.

¹⁴ Underhill. *This Journal*, 11, p 485, 1906-07, see also Underhill and Rand. *Arch of Int Med*, 1, p 61, 1911.

TABLE 5
Dog 52—Inanition

DATE	DAILY DOSE OF COCAINE	BODY WEIGHT	URINE				
			Volume	Specific Gravity	Total Nitrogen	Ammonia Nitrogen	Lactic Acid
	mgms	kilos	cc		grams	gram	mgms
1910 November							
10	120	10.2	160	1.050	6.57	0.31 (4.7)*	41
11	120	10.2	120	1.058	4.23	0.39 (9.2)	38
12	120	9.9	180	1.025	3.06	0.34 (11.1)	39
13	120	9.6	140	1.035	2.73	0.26 (9.5)	36
14	120	9.3	200	1.040	4.92	0.31 (6.3)	32
15	150	9.0					
	120						
16	2 x 150	8.9	70	1.030	1.80	0.13 (7.3)	13
17	2 x 150	8.8	160	1.030	3.60	0.25 (6.9)	21
18	2 x 150	8.6	100	1.018	0.48	0.03 (6.2)	5
19	2 x 150	8.5	100	1.020	3.25	0.12 (3.6)	25

* Figures in brackets indicate percentages of total nitrogen

which accompanies this pathological state. The observations noted above are also opposed to the results obtained in phosphorus poisoning¹⁵ a condition in which carbohydrate is almost missing from the liver and blood. On the other hand, hydrazine¹⁶ which behaves in a manner similar to phosphorus with respect to its influence upon the carbohydrate of the organism does not lead to the appearance of appreciable quantities of lactic acid in the urine. From these contradictory results it is apparent that lactic acid must have a diverse origin under the different conditions mentioned. The ammonia content of the urine voided by the dog in a state of inanition was not greatly influenced by the cocaine injections and did not bear a direct relationship to the elimination of lactic acid.

¹⁵ Frank and Isaac *Arch f exp Path u Pharm*, liv, p 374, 1911

¹⁶ Underhill *This Journal*, x, p 159, 1911

From the observations here recorded the conclusion may be drawn that the appearance of lactic acid in increased quantity during cocaine poisoning is probably associated with the attendant increased muscular activity induced by the action of the drug upon the nervous system. What relation augmented lactic acid output bears to lack of oxygen as claimed by Araki is a problem difficult of decision unless one accepts the view put forth by Feldman and Hill¹⁷ that increased muscular work results in a decreased amount of oxygen in the muscles, which in turn causes an increased production and subsequent excretion of lactic acid.

It is also apparent that in cocaine poisoning greater quantities of lactic acid are eliminated by given doses of cocaine to well-fed animals than occurs under the same conditions during an interval of starvation. The average elimination of lactic acid during cocaine poisoning in a state of inanition was less than that of other animals maintained in a well-fed condition, but without cocaine administration. It seems probable, therefore, that during cocaine poisoning, carbohydrate material may be intimately associated with the production of lactic acid.

CONCLUSIONS

In confirmation of previous investigation, it is found that cocaine introduced subcutaneously into dogs causes a temporary but significant increase in body temperature.

With daily doses of 10 mgms of cocaine hydrochloride per kilo of body weight for short periods of time no influence can be detected upon nitrogenous metabolism nor upon fat utilization.

Fat utilization is slightly impaired and body weight is considerably decreased when daily injections of 15 mgms cocaine are administered.

When the dose of cocaine is increased to 20 mgms per kilo body weight per day a distinct lowering of both nitrogen and fat utilization is noted. This may be accompanied by a slight negative nitrogen balance.

Lactic acid excretion in the urine is markedly increased in well-fed dogs and rabbits as a result of cocaine injection. In a starving

¹ Feldman and Hill *loc cit*

condition the dog eliminates less lactic acid after cocaine injections than is excreted by the normal well-fed animal

It is not unlikely that the increased lactic acid elimination after cocaine injections is associated with increased muscular activity induced by the drug

The ammonia output apparently bears little relation to lactic acid elimination under the experimental conditions

Lactic acid and carbohydrate metabolism are presumably intimately associated although there are indications that lactic acid may at times arise from more than a single antecedent

ON CREATINE IN THE URINE OF CHILDREN

By OTTO FOLIN AND W DENIS

(*From the Biochemical Laboratory of Harvard Medical School, Boston*)

(Received for publication, February 29, 1912)

In a recent communication from Mendel's laboratory¹ Rose showed by means of an extended series of analyses that the urine of children usually contains relatively large quantities of creatine. These observations are remarkable because they apply only to children and do not correspond to what is found in older people. In adults creatine is believed to be eliminated only when much creatine is taken with the food or when there is an unusual disintegration of tissue materials—a condition more or less the reverse of that prevailing in growing children.

In view of the unexpected character of the results obtained by Rose we promptly repeated the work on three normal well nourished children belonging to one of us (Folin). We had intended to continue the investigation further before publishing anything, but in view of the criticism of Rose's findings expressed by Wolf² and by McCrudden³ and since our results completely verify and also extend Rose's observations we have decided to publish them now.

The subjects are Joanna, age 11, weight 38 K, George, age 8 years, 8 months, weight 33.5 K, Teresa, age 3 years, 8 months, weight 16 K.

If the appearance of creatine in the urine of children were essentially the result of carbohydrate starvation one would expect the night urine to be particularly rich in creatine.

¹ This *Journal*, x, p 265, 1911

² This *Journal*, x, p 473, 1912

³ *Journ of Exp Med*, xv, p 110, 1912

EXPERIMENT 1 The night urine obtained on the morning of October 17, 1911, yielded per 100 cc of urine the figures recorded below (The children had had some meat the preceding noon but none for supper)

NAME	CREATININE	CREATINE
	<i>mgm</i>	<i>mgm</i>
1 J	52 0	19 0
2 G	89 0	22 0
3 T	87 5	9 7

EXPERIMENT 2 The afternoon urines obtained from the same children about a week later gave the following figures (per 100 cc of urine)

NAME	CREATININE	CREATINE
	<i>mgm</i>	<i>mgm</i>
1 J	76 5	46 5
2 G	66 0	15 0
3 T	16 3	8 7

In order to find out whether any hourly variation is present we next determined the creatinine and creatine for each hour from 9 in the morning till 3 in the afternoon in the urine obtained from one of the subjects, J The results are recorded in Experiment 3

EXPERIMENT 3 Breakfast 9 a m (no meat), Dinner 1 15 p m, including meat

TIME	VOLUME	CREATININE	CREATINE
	<i>cc</i>	<i>mgm</i>	<i>mgm</i>
9-10	47	34 8	3 7
10-11	32	28 8	7 7
11-12	30	25 2	9 0
12-1	40	28 0	8 0
1-2 15	80	30 4	3 6
2 15-3 15	98	24 1	12 0

The most striking point to be observed in the above results is the sharp and decisive rise in the creatine output, presumably as a result of eating meat at dinner The breakfast also, although it included no meat is followed by a rise rather than by a fall in the creatine output

We are inclined to believe that the creatine in children's urine does not depend as Rose suggests on a peculiar carbohydrate metabolism but that it is due to an excessively high level of protein consumption (in proportion to mass of muscles in the body) We know that the creatine output in response to creatine feeding depends very much on the level of protein metabolism maintained Whether the creatine is taken with the food, as in the experiments of Folin and of Klercker, or whether it comes directly from the tissues, as in fevers (and possibly also in starvation) may be more or less immaterial in view of the fact ascertained by us⁴ that creatinine and creatine like urea and amino acids⁵ are promptly transported from the digestive tract to the blood and from the blood to the tissues We hope later to prove (or disprove) experimentally the validity of this point of view⁶ In this paper we wish merely to corroborate Rose's interesting findings as to the fact that creatine is nearly always found in the urine of children

EXPERIMENT 4 Twenty-four hour urine on a mixed diet including some meat at noon

NAME	VOLUME	TOTAL NITROGEN	CREATININE	CREATINE
	cc	grams	mgm	mgm
J	1340	10.1	643	258
G	1000	11.6	810	90
T	680	6.2	219	186

⁴ The absorption experiments with creatine and creatinine will be described in detail later The creatinine we have traced from the intestine through the blood to the tissues by means of colorimetric creatinine estimations as well as by nitrogen determinations

⁵ This *Journal*, xi, p. 87, 1912

⁶ If the above hypothesis is correct it should be possible to reproduce in adults by forced feeding with protein which contains no creatine the condition with reference to creatine found in children and it should also be possible to obtain creatine free urine from children by reducing their protein consumption

EXPERIMENT 5 Twenty-four hour urine from mixed diets containing no creatine

NAME	DAY	VOLUME	TOTAL NITROGEN	CREATININE	CREATINE
		<i>cc</i>	<i>grams</i>	<i>mgm</i>	<i>mgm</i>
J	First	1050	7 8	720	160
J	Second	1085	7 8	399	140
J	Third	955	7 9	477	86
G	First	790	8 6	620	100
G	Second	1330	10 6	385	119
G	Third	1075	11 45	481	150
T	First	850	4 9	190	90

EXPERIMENT 5 Night urine from Dr A H Wentworth's children, Elizabeth, age 9 years, 6 months, and Charles, age 6, on a creatine-free diet. The figures are given for 100 cc of urine

NAME	DAY	NITROGEN	CREATININE	CREATINE
		<i>grams</i>	<i>mgm</i>	<i>mgm</i>
E	Second	0 86	34	3
E	Third	0 85	34	3
E	Fourth	1 17	36	4
C	Second	0 96	31	4
C	Third	0 75	24	6
C	Fourth	0 96	30	4

EXPERIMENT 6 Through the kindness of Professor Wiener we are able to include in our determinations the morning urine obtained from his four healthy and unusually robust children, all of whom are vegetarians and have never eaten any food containing creatine —Norbert, age 17 years, 3 months, Constance, age 13 years, 10 months, Bertha, age 9 years, 10 months, Fritz, age 6. As before the figures are given for 100 cc of urine

NAME	TOTAL NITROGEN	CREATININE	CREATINE
	<i>grams</i>	<i>mgm</i>	<i>mgm</i>
N	1 1	115	18
C	0 7	50	8
B	0 9	55	10
F	0 5	24	11

A NEW METHOD FOR THE DETERMINATION OF HIPPURIC ACID IN URINE

By OTTO FOLIN AND FRED F FLANDERS¹

(From the Biochemical Laboratory of Harvard Medical School, Boston)

(Received for publication, February 29, 1912)

Bunge and Schmiedeberg's well known method for the determination of hippuric acid in urine was published in 1876. That method is neither accurate nor convenient. It has survived evidently only because no one has succeeded in devising anything better. The more recent methods which have been proposed from time to time have been only modifications of that method. They retain the tedious extraction by means of acetic ether and depend for their accuracy on the isolation of perfectly pure hippuric acid.²

Benzoic acid is less soluble in water and much more soluble in organic solvents than is hippuric acid. The quantitative extraction of benzoic acid and its determination by direct titration in the organic solvent (chloroform) is a relatively simple, convenient and exact process for the determination of benzoic acid,³ in products far more difficult to handle than urine. If hippuric acid could be conveniently hydrolyzed into benzoic acid and glycocoll the determination of hippuric acid in urine might be made almost as simple as the determination of benzoic acid. In our attempt to work out a method for determining hippuric acid according to this scheme we met with many unforeseen difficulties and some surprises but the final outcome is, we believe, reasonably satisfactory.

While it is generally recognized that it is possible to split hippuric acid by either acids or alkalis, the former are in practice

¹ Published with the approval of the committee as work done under a Bullard Fellowship, 1911-1912.

² For the most recent modification see Dakin. *This Journal*, vii, p 103, 1910.

³ Folin and Flanders. *Journ of the Amer Chem Soc*, xxxiii, p 161, 1911.

uniformly preferred for that purpose. In fact hippuric acid is tacitly assumed to be more stable in weakly alkaline than in neutral or acid solutions, for in preparing hippuric acid from urine some alkali (calcium hydrate or sodium carbonate) is generally added before the urine is concentrated. Definite data on the subject we have not been able to find.⁴ It has not been our aim to furnish such data because our purpose was to accomplish the quantitative hydrolysis of hippuric acid in urine under conditions that would permit a rapid and convenient extraction of the benzoic acid from the resulting mixture. In other words emulsion with the organic solvent used for the extraction was with us the most serious obstacle to be avoided and the hydrolysis had to be made with that end in view. Incidentally we have, however, ascertained a few specific facts as to the stability of hippuric acid which are worth recording. They are contained in the table below.

WEIGHT OF HIPPURIC ACID	TOTAL VOLUME	HYDROLYZING AGENT	TIME AND TEMPERATURE	HIPPURIC ACID HYDROLYZED	
				gram	per cent
gram	cc				
0.2	50	0.01 gm NaOH	16 hrs on water bath	0.0058	2.9
0.2	50	0.025 gm NaOH	16 hrs on water bath	0.0066	3.3
0.2	50	0.05 gm NaOH	16 hrs on water bath	0.0116	5.8
0.2	50	0.25 gm NaOH	16 hrs on water bath	0.1966	98.2
0.2	50	0.2 gm Na ₂ CO ₃	16 hrs on water bath	0.0117	5.8
0.2	50	2.0 gm Na ₂ CO ₃	16 hrs on water bath	0.0208	10.4
0.2	50	2 gms urea	16 hrs on water bath	None	None
0.2	50	0.5 gm acetic acid	16 hrs on water bath	Trace	
0.2	150	Excess of milk of lime	Boiled $\frac{1}{2}$ hr	Trace	
0.2	100	Excess of milk of lime	Boiled 3 $\frac{1}{2}$ hrs	0.0092	4.6
0.03	30	4.5 gms HCl	Boiled 1 $\frac{1}{2}$ hrs	0.027	90.0
0.15	75	11.4 gms HCl	Boiled 1 $\frac{1}{2}$ hrs	0.1323	88.2
0.15	70	18 gms HNO ₃	Boiled 1 $\frac{1}{2}$ hrs	0.1242	82.8

⁴ See, however, Dessaignes *Journ Pr Chem*, (1) xxxii, p 244, 1846

WEIGHT OF HIPPURIC ACID	TOTAL VOLUME	HYDROLIZING AGENT	TIME AND TEMPERATURE	HIPPURIC ACID HYDROLIZED	
				gram	per cent
0 15	70	18 gms HNO_3	Boiled 3 hrs	0 1280	85 3
0 15	70	18 gms HNO_3	Boiled 8 hrs	0 1303	86 8
0 15	60	9 mgs HNO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled $1\frac{1}{2}$ hrs	0 0620	41 3
0 15	60	9 gms HNO_3 + 0 2 gm Hg	Boiled $1\frac{1}{2}$ hrs	0 0240	16 0
0 15	60	9 gms HNO_3 + 35 gms NaNO_3	Boiled $1\frac{1}{2}$ hrs	0 620	41 3
0 15	60	9 gms HNO_3 + 35 gms NaNO_3	Boiled 3 hrs	0 1395	93 0
0 15	70	18 gms HNO_3 + 1 gm $\text{Cu}(\text{NO}_3)_2$	Boiled $1\frac{1}{2}$ hrs	0 1257	83 8
0 15	70	18 gms HNO_3 1 gm $\text{Cu}(\text{NO}_3)_2$ 35 gms NaNO_3	Boiled $1\frac{1}{2}$ hrs	0 1411	94 1
0 15	70	18 gms HNO_3 + 0 2 gm Hg	Boiled 3 hrs	0 1227	81 8
0 15	70	18 gms HNO_3 35 gms NaNO_3	Boiled 3 hrs	0 1457	97 1
0 15	70	18 gms HNO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled 3 hrs	0 1441	96 0
0 15	70	18 gms HNO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled 4 hrs	0 1500	100
0 05	50	23 gms HNO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled $4\frac{1}{2}$ hrs	0 0504	100 8
0 10	50	23 gms HNO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled $4\frac{1}{2}$ hrs	0 1000	100
0 15	50	23 gms HNO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled $4\frac{1}{2}$ hrs	0 1487	99 1
0 2	50	23 gms NHO_3 + 0 2 gm $\text{Cu}(\text{NO}_3)_2$	Boiled $4\frac{1}{2}$ hrs	0 1973	98 7

The figures given in the above table show that while it is possible to split hippuric acid quantitatively by boiling with mineral acids the treatment required for this purpose is rather heroic. The quantitative decomposition is much more easily accomplished by means of alkalis and there can hardly be any doubt but that hippuric acid is much less decomposed in the presence of dilute acids than in the presence of small amounts of alkali. In view of these findings it is clearly a mistake to render urine alkaline before

concentrating it when preparing hippuric acid from urine and still more so when the hippuric acid is to be extracted for quantitative determinations. The lack of agreement among investigators on the transformation of benzoic acid to hippuric acid in the animal body is doubtless due in part at least, to losses of hippuric acid by its transformation back into benzoic acid in the urine, during the concentration of the latter.

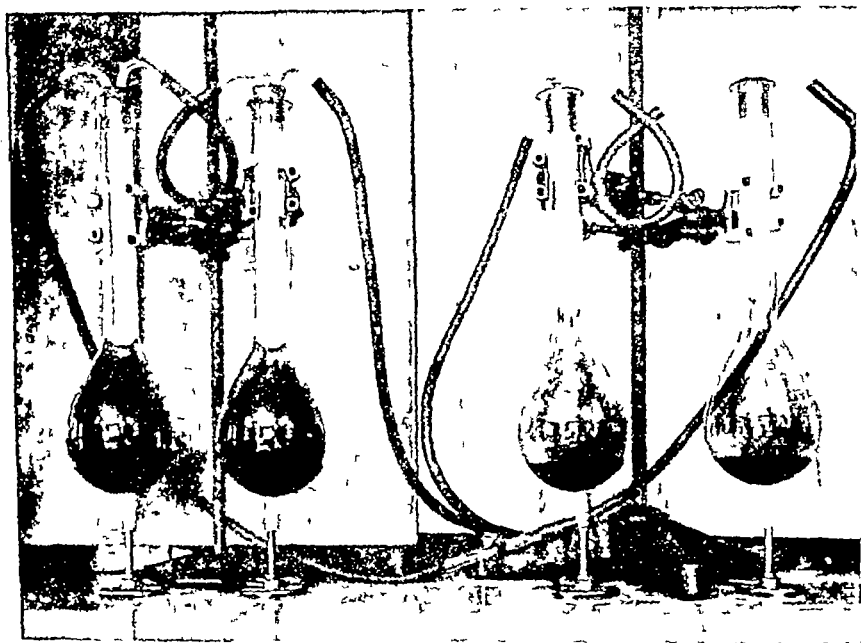
In our method the hydrolysis of the hippuric acid is on the other hand an essential feature. In fact we lost much time in experimenting with various acids, catalyzers, oxidizing reagents, etc., to bring about complete hydrolysis before we discovered that the greater part of the hippuric acid is split while the urine is being concentrated on the water bath. After having discovered such a convenient and effective method for splitting the hippuric acid it would seem that one should be able to merely acidify the urine and at once extract with chloroform. Ultimately a way will doubtless be found to do this but so far we have been unable to accomplish it satisfactorily. The extraction of the benzoic acid with chloroform is neat, clean, rapid and complete only when it is not complicated by emulsions. The best way which we have found to eliminate the emulsion and the coloring matters of the urine is to boil the urine for several hours with comparatively strong nitric acid.

The method in detail, as finally adopted, is as follows. Measure 100 cc. of urine into a porcelain evaporating dish by means of a pipette. Add 10 cc. of 5 per cent NaOH and evaporate to dryness on the steam bath. (If the sample is placed on the bath at night it will be dry in the morning.) Transfer the residue to a 500 cc. Kjeldahl flash by means of 25 cc. of water, and 25 cc. of conc. HNO_3 . Add 0.2 gram copper nitrate, a couple of pebbles or glass pearls and boil very gently four and one-half hours over a microburner.

The necks of the flasks are fitted with Hopkins condensers, made from large test tubes which fit rather loosely. A good current of water flowing through the condensers prevents loss of benzoic acid or change in concentration of the nitric acid. The accompanying photograph shows the arrangement of the apparatus.

After cooling the condensers are rinsed down with 25 cc. of water, and the contents of the flask are transferred to a 500 cc.

separatory funnel by the use of 25 cc more of water The total volume of the solution is now 100 cc Add to the solution sufficient ammonium sulphate to just saturate it (about 55 grams) Make four extractions with freely washed chloroform, using 50, 35, 25 and 25 cc portions The first two portions may be used to further rinse out the Kjeldahl flask The separatory funnels may be shaken vigorously as there is practically no tendency to form an emulsion



The successive portions of chloroform are collected in another separatory funnel Add to the combined extracts 100 cc of saturated solution of pure sodium chloride, to each liter of which has been added 0.5 cc of concentrated HCl Shake well, draw the chloroform into a dry 500 cc Erlenmeyer flask and titrate with $\frac{N}{10}$ sodium alcoholate, using four or five drops of phenolphthalein as indicator The first distinct end point should be taken, although it may fade on standing a short time

The sodium ethylate is made by dissolving 2.3 grams of cleaned metallic sodium in one liter of absolute alcohol ⁵ It is advisable that it be slightly weaker rather than stronger than tenth-normal

It may be standardized against purified benzoic acid in washed chloroform, or with certain restrictions against tenth-normal hydrochloric acid in aqueous solution In a recent contribution, it was stated that the value found by titration in aqueous solution was slightly higher than that found by the chloroform ⁶ The cause of this variation has been traced to sodium carbonate, which is formed by the absorption of carbon dioxide The point has an interesting theoretical, as well as practical side A rather large quantity of the ethylate was gradually used with frequent opening over a period of three months As it was nearly exhausted, quite a precipitate was noticed in the bottom of the bottle At this juncture titrations were made against acid solutions of equivalent normality in order to test the standard

The results appeared as follows

Ten cubic centimeters $\frac{N}{20}$ oxalic acid in chloroform required 5.55, 5.6 and 5.6 cc of the ethylate

Ten cubic centimeters $\frac{N}{20}$ hydrochloric acid in aqueous solution required 5.4, 5.4, 5.4 cc of the ethylate The ethylate was filtered, after which it was not quite transparent, but freed from nearly all the precipitated carbonate The titrations were repeated with results as follows

Ten cubic centimeters $\frac{N}{20}$ oxalic acid in chloroform required 5.6 and 5.65 cc ethylate Ten cubic centimeters $\frac{N}{20}$ aqueous oxalic acid required 5.52, 5.55 and 5.58 cc sodium ethylate

Ten cubic centimeters $\frac{N}{20}$ aqueous hydrochloric required 5.55 and 5.55 cc ethylate

To further emphasize the point, the same quantities of $\frac{N}{20}$ oxalic solution in chloroform were titrated after adding 0.1 gram of dry sodium carbonate to each 10 cc $\frac{N}{20}$ oxalic acid required 5.5 and 5.6 cc of the ethylate From this it is plain that the sodium carbonate does not influence the titration in chloroform, but of course does materially affect the aqueous titrations

The following results may be cited as showing the agreement in duplicates obtainable by this method We believe that they are

⁵ This *Journal*, vii, p 423

⁶ *Journ Amer Chem Soc*, xxxiii, p 1625, 1911

more nearly correct than are the figures obtainable by any other method

NO	HIPPURIC ACID TITRATION	VOLUME OF URINE	TOTAL
	cc	cc	
1	5 30	800	0 764
	5 45		0 786
2	8 55	1,040	1 602
	8 65		1 622
3	4 65	1,180	0 990
	4 70		1 000
4	5 30	1,200	1 148
	5 35		1 158
5	7 00	610	0 770
	6 90		0 760
6	5 30	870	0 832
	5 25		0 825
7	8 60	700	1 086
	8 30		1 049
8	14 6	730	1 920
	14 4		1 880

ON THE BLUE COLOR REACTION OF PHOSPHOTUNGSTIC ACID (?) WITH URIC ACID AND OTHER SUBSTANCES

(PRELIMINARY PAPER)

BY OTTO FOLIN AND A B MACALLUM

(From the Biochemical Laboratory of Harvard Medical School, Boston, Mass.)

(Received for publication, March 19, 1912)

The beautiful blue color which is produced when phosphotungstic acid and an alkali are added to uric acid lends itself unusually well to quantitative work. After several months spent in trying to devise a direct method for a colorimetric determination of uric acid in urine on the basis of this reaction, we have reluctantly come to the conclusion that this is not feasible, because of the presence in urine of substances other than uric acid which give the same reaction. In uric acid solutions the colorimetric values obtained are sharply proportionate to the amount of uric acid present, and the color fades so slowly when the conditions are right that we do not hesitate to pronounce the reaction eminently suitable for the determination of small quantities of uric acid. The reaction is almost instantaneous and the color remains practically unchanged for almost ten minutes, so that no difficulty is experienced in making the necessary quantitative comparisons by means of a colorimeter. To secure the maximum color of the desired stability the strong alkalis usually employed in making the reaction can not be used, a saturated solution of sodium carbonate is very much better.

In the course of our further studies we have discovered that the color in question is given not only by uric acid but is characteristic of phenols, and that in the case of more complex aromatic compounds it is particularly, if not exclusively, those containing a hydroxyl group in the para position which give the color. This discovery has of course given a new turn to our investigations.

We believe that the reaction will be found fully as useful as Millon's for the detection of certain aromatic groups in protein substances, and that it has the advantage of being particularly suitable for quantitative work. Among the substances which give the reaction may be mentioned phenol, tyrosine, tannic acid, thymol, orcin, resorcin, vanillin, and phloroglucin, besides a number of less definite protein materials.

A more detailed study of this interesting reaction and its application for the detection and determination of such aromatic products will be undertaken as soon as we get through with the uric acid work. The best procedure which we have yet found for the determination of uric acid in urine is to precipitate the uric acid by means of silver sulphate and magnesia mixture, centrifuge, and make the color reaction on the precipitate in the presence of formaldehyde. (The latter is added to reduce the silver.)

It would be useless to describe the method in detail at the present time, for we have found that different samples of phosphotungstic acid (and phosphomolybdic acid) do not produce the same intensity of color. In fact the material which produces the blue color with uric acid and with phenols is probably not phosphotungstic acid. Whether it is a tungsten product at all, or some other substance present as impurity, we have not yet been able to determine for lack of material. We have learned how to concentrate the active agent and to separate it from the greater part of the phosphotungstic acid, but more material and more work will be required before we shall know what it is and how to get it free from the useless, as well as expensive, phosphotungstic acid.

STUDIES IN THE ACTION OF TRYPSIN

I ON THE HYDROLYSIS OF CASEIN BY TRYPSIN

By E. H. WALTERS

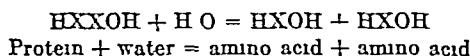
(From the Rudolph Spreckels Physiological Laboratory of the University of California)

(Received for publication, February 28, 1912)

I INTRODUCTION

(a) *Objects of the investigation*

It is a well known fact that the digestion of protein is essentially a process of hydrolysis. This reaction may be represented by the following schematic equation



The mode of operation of this reaction is entirely beyond the category of our present knowledge. The researches of Emil Fischer¹ on the hydrolysis of the synthetic polypeptides will, it is hoped, throw some light on this question, for many of these synthetic products are known to be hydrolyzed by trypsin.

It has been found that this reaction proceeds at all temperatures in neutral watery solutions free from a ferment or any other catalyser except, possibly, the ions of the water itself. Taylor² working with sterile solutions of casein, protamine sulphate, and nucleoprotein in pure water and Robertson³ working with casein have demonstrated directly that these bodies are hydrolyzed at

¹ Emil Fischer. Numerous papers in the *Berichte der deutsch chem Gesellsch*, and in the *Zeitschr f physiol Chem* during the past decade.

² Taylor. On Fermentation, *Univ Calif Pub Pathol*, 1, p 97, 1907.

³ T. Brailsford Robertson. The Proteins, *Univ Calif Pub Physiol*, III, p 174, 1909 (see footnote).

ordinary temperatures Robertson found that comparatively rapid hydrolysis occurs when neutral solutions of the caseinates in pure sterile water are kept at 36°C . For a 2.8 per cent solution of neutral sodium caseinate the velocity constant (common logarithms being employed and time expressed in hours) was found after twenty days at 36°C to be 0.000518. These observations are enough to show that a protein is not in equilibrium with its products.

Kühne⁴ pointed out that the portion of the albumin molecule which is readily dissociated by the action of trypsin contains a high percentage of tyrosine and tryptophane, while the portion which was not acted upon by trypsin is characterized by the presence of glycocoll and a carbohydrate radical. The high tyrosine and tryptophane contents and the absence of glycocoll and a carbohydrate radical renders casein especially readily digestible. The action of trypsin on caseinates in neutral or faintly alkaline solution is, therefore, one of the best examples among protein reactions of the action of an enzyme in accelerating an already progressing chemical transformation.

Observations already made on the mode of the above reactions when accelerated by a proteolytic ferment and to which references will be made *in loco* indicate that the process of the hydrolysis of proteins by trypsin or pepsin obeys the monomolecular reaction formula during the initial stages of the reaction when the influence of the products of hydrolysis is practically nil.

This research was undertaken to determine, under more exactly defined conditions than hitherto, the influence of certain factors upon the hydrolysis of proteins by trypsin. The objects of the investigation are three-fold, firstly, to ascertain the relation between the time of hydrolysis and the amount of protein hydrolyzed, secondly, to ascertain the relation between the ferment concentration and the velocity of hydrolysis for different concentrations of protein, and thirdly, to determine the relation between the nature of the base combined with a protein and the velocity with which it is hydrolyzed.

Rigidly comparable solutions of the caseinates can very readily

⁴ Kühne quoted after Mann, *Chemistry of the Proteins*, Macmillan Co., London, 1906, p. 148.

be prepared,⁵ and in view of the useful properties of casein in studying the process of the hydrolysis of a protein by an enzyme, the ease with which it is attacked by trypsin, its accurate quantitative estimation, and since the action of trypsin on casein has already been studied somewhat extensively, I have held to this system in the present investigation

(b) *Influence of antiseptics*

It is obvious that in experiments of this character the solutions under investigation must be kept sterile by the action of an antiseptic which does not alter the course of the reaction or impede the action of the ferment. It has been found by different observers that toluol satisfies the conditions very satisfactorily. Weis⁶ has found that toluol does not sensibly affect the action of trypsin or pepsin when used in just the quantities necessary to maintain sterility. On the contrary thymol, chloroform, formol, benzoic acid and salicylic acid slightly retarded the enzyme action. Kaufmann⁷ found that toluol, chloroform, thymol, and sodium fluoride destroyed the action of trypsin after a considerably long period of time, after twenty-four hours' action, the most concentrated solutions of the ferment were rendered inactive. This effect, however, is not entirely due to the action of the antiseptic as it is well known that trypsin is very readily rendered inactive on standing in pure water, although the destruction of the ferment may be accelerated in the presence of an antiseptic. Bayliss⁸ found that toluol slightly accelerated the action of trypsin during the course of an experiment and that chloroform had no perceptible influence. I used toluol in all of my experiments in the proportion of 0.2 cc to 100 cc of casein solution as it was found that 100 cc of a 2 per cent solution of "basic" sodium caseinate containing this amount of antiseptic were still sterile after the lapse of three weeks. No systematic study was made to determine the influence of the antiseptic but according to Weis the quantity used would not alter the reaction or the activity of the enzyme.

⁵ T Brailsford Robertson *Journ of Physical Chem*, xiv, p 377, 1910

⁶ Weis *Compt rend des trav du lab de Carlsberg*, v p 133, 1900

⁷ Kaufmann *Zeitschr f physiol Chem*, xxix, p 434, 1903

⁸ Bayliss *The Kinetics of Tryptic Action*, *Arch sci biol* (St Petersburg, 1904), 11 Suppl, p 261, reprinted in the *Collected Papers of the Physiological Laboratory, University College, London*, viii, 1903-5

(c) *The influence of alkalis and acids upon digestion by trypsin*

It is now generally recognized that trypsin acts most energetically in faintly alkaline solutions although the evidence upon which this fact rests is extraordinarily at variance. According to Heidenhain⁹ the action of definite percentages of Na_2CO_3 varies with the quantity of the ferment, but for moderate concentrations the activity is most pronounced in solutions of 0.9 to 1.2 per cent Na_2CO_3 . He also pointed out that the addition of 0.1 per cent HCl to an aqueous extract of the pancreas entirely stops its action. Kuhne¹⁰ found that trypsin acts in HCl solutions up to 0.05 per cent, above which its activity ceased altogether. In his experiments, however, its action was most pronounced in solutions of 0.3 per cent Na_2CO_3 . Mays¹¹ and Ewald¹² also found that trypsin could digest fibrin in the presence of 0.3 per cent HCl only when large amounts of the protein were present, and Mays confirmed the statement of Kuhne that trypsin is destroyed by pepsin and HCl . On the other hand, Engesser's¹³ experiments show that pancreatic juice did not lose its digestive power by two hours warming with a gastric juice containing 0.5 per cent HCl . Langley¹⁴ conducted a series of experiments which show that a glycerine extract of the pancreas when warmed for two and a half hours in solutions of 0.05 per cent HCl decreases considerably in its digestive power, a result diametrically opposed to that of Engesser's. Lindberger¹⁵ found that fibrin was very slowly dissolved by trypsin in the presence of 0.012 per cent HCl and that the action of the ferment entirely ceased in the presence of 0.1 per cent HCl . He also observed that weaker acids, as acetic and lactic, had a much less retarding effect than the stronger HCl and that tryptic digestion was rapid and in some

⁹ Heidenhain *Pflüger's Archiv*, v, p 557, 1875

¹⁰ Kuhne *Verh Naturhist med Vereins zu Heidelberg*, 1877, p 193, quoted after Chittenden and Cummins, *Amer Chem Journ*, vii, p 36, 1885

¹¹ Mays *Untersuch a d physiol Institut in Heidelberg*, iii, p 378, 1880, quoted from *Maly's Jahresbericht*, v, p 299, 1880

¹² Ewald *Zeitschr f klin Med*, i, p 615, quoted from *Maly's Jahresbericht*, v, p 297, 1880

¹³ Engesser *Zeitschr f klin Med*, ii, p 192, quoted from *Maly's Jahresbericht*, v, p 297, 1880

¹⁴ Langley *Journ of Physiol*, iii, p 246, 1880

¹⁵ Lindberger *Maly's Jahresbericht*, viii, p 280, 1883

cases even more energetic in the presence of small quantities of these acids than in neutral solutions

Chittenden and Cummins¹⁶ found that the addition of Na_2CO_3 to 0.2 per cent increased the tryptic digestion of fibrin and between 0.2 and 0.5 per cent Na_2CO_3 the action was about the same and above 0.5 per cent the action was greatly retarded. These authors also found that very small amounts of hydrochloric and salicylic acids greatly retard the action, its proteolytic action being retarded to a minimum before any free acid is present. Three-tenths per cent combined HCl has a great retarding effect, and the same amount of combined salicylic acid plus 0.1 per cent free salicylic acid produces similar results. Much smaller quantities of combined salicylic acid (0.06 per cent) have the same effect. Combined hydrochloric acid has a greater hindering action than salicylic acid.

Vernon¹⁷ observed that trypsin is very rapidly destroyed in 0.4 per cent solutions of Na_2CO_3 . His results indicate that a series of "trypsins" might exist as different preparations were unequally affected by constant amounts of sodium carbonate, the least sensitive ones being more resistant to the action of the alkali. The preparations were also more resistant to the action of sodium carbonate in the presence of large amounts of protein. This statement was subsequently confirmed by Bayliss and Starling.¹⁸ Schierbeck¹⁹ states that carbonic acid augments the action of trypsin in alkaline solutions since it diminishes the alkalinity of the solution.

According to Bayliss,²⁰ Kamitz,²¹ Taylor,²² Robertson and Schmidt,²³ and Kudo²⁴ the influence of alkalies and acids is due to

¹⁶ Chittenden and Cummins *Amer Chem Journ*, vii, p 36, 1885

¹⁷ Vernon *Journ of Physiol*, xxvi, p 427, 1900

¹⁸ Bayliss and Starling *Journ of Physiol*, xxxii, p 129, 1905

¹⁹ Schierbeck *Stand Arch f Physiol*, ii, p 344, 1892

²⁰ Bayliss The Kinetics of Tryptic action, *Arch sci biol* (St Petersburg, 1904) 11 Suppl, p 261, reprinted in the *Collected Papers of the University College Physiological Laboratory, London*, xii, 1903-5

²¹ Kamitz *Zeitschr f physiol Chem*, xxxvii, p 75, 1902

²² Taylor On Fermentation, *Univ of Calif Pub Pathol*, i, p 251, 1907

²³ T Brailsford Robertson and C L A Schmidt *This Journal*, v, p 31, 1908

²⁴ Kudo *Biochem Zeitschr*, xv, p 473, 1909

the OH and H ions since it is recognized that in most cases the acids and alkalies act in proportion to their degree of dissociation. Thus Kanitz has observed from the results of Dietz²⁵ that the action of the hydroxides of Ca, Sr, and Ba upon tryptic digestion is a function of their degree of dissociation and that the optimum OH ion concentration lies between $\frac{1}{100}$ and $\frac{1}{1000}$ normal. This is an observation almost identical with that of Vernon's²⁶ although his result is expressed in terms of sodium carbonate as per cent. In experiments on the digestion of protamine by trypsin Taylor learned by the aid of the gas cell that the most favorable initial concentration of alkali is that which is sufficient to neutralize about $\frac{1}{1000}$ acid solution after neutralization of the products of the hydrolysis, which are slightly acid. Kudo has found that trypsin acts best in neutral solutions and is inhibited by acids and alkalies in proportion to their degree of dissociation into H and OH ions. Robertson and Schmidt made an investigation to determine the part played by the alkali in digestion by trypsin. Sodium caseinate and protamine sulphate were used as substrate and the alkalinities of the digests were followed throughout the digestion and determined by means of the gas-chain. It was found that the change in OH⁻ concentration with time obeyed the monomolecular formula for all alkalinities above 10^{-6} normal and for concentrations less than this the velocity of the reaction diminished and the bimolecular formula held good. Moreover, this value, 10^{-6} N OH⁻, at which the order of the reaction changes is independent of the nature of the protein or the initial concentration of the alkali. It was concluded, therefore, that all alkalinities between $\frac{1}{1000000}$ and about $\frac{1}{1000}$ are equally favorable for tryptic action. The latter value $\frac{1}{1000}$ N, as we have seen, is the one determined by Taylor.

On the other hand the results of Berg and Gies²⁷ do not support this view although it was recognized that the H and OH ions were the favorable acid and alkali factors. No regular results were obtained in equivalent solutions of different bases and it appeared

²⁵ Dietz. *Einfluss von Baryumoxyhydrat, Calciumoxyhydrat, Strontiumoxyhydrat auf die tryptische Verdauung*, Inaug. Dissertation, Leipzig, 1900.

²⁶ *Loc. cit.* For the calculation of this value consult Shield. *Zeitschr. f. physik. Chem.*, 11, p 167, 1893.

²⁷ Berg and Gies. *This Journal*, 11, p 489, 1906.

that the cations or molecules (or both) exercised deterrent influences

Loeb²⁸ explains the accelerating action of alkalis in tryptic digestion by assuming that the enzyme is a weak acid and upon the addition of alkali a salt is formed which is more strongly dissociated than the acid itself. This latter is based upon the fact that salts of weak bases and acids are more highly dissociated than the free bases and acids themselves. If the enzyme action is, therefore, due to the enzyme ion, its acting mass will be greater in the presence of enzyme salts.

In my experiments the initial OH^- concentrations were under rigid control. The solutions were made so that the proportion of base to casein = 80×10^{-5} equivalents per gram. These solutions are neutral to phenolphthalein, *i.e.*, faintly alkaline, the OH^- concentration being 10^{-5} N .²⁹

(d) *Method of measurement*

Most of the conflicting data on the hydrolysis of proteins has resulted largely from the inaccuracy of the measurements. In most of the investigations the mode of measurement has not been adequate to estimate the actual transformation since in nearly all cases the errors in the methods have ranged from 10 to 50 per cent. In the majority of the studies some alteration in the physical properties of the solutions such as electrical conductivity, viscosity, osmotic pressure, optical activity, etc., have been used as a means of determining the degree of hydrolysis. Moreover, the chemical methods that have been employed have been based upon more or less empirical estimates in which the substances measured bore no direct chemical relation to the amount of substance undergoing transformation. These methods will be alluded to throughout the paper in connection with the experiments in which they were employed.

The most reliable of the properties of the proteins for obtaining constants characteristic of them is undoubtedly the number representing the quantity of nitrogen bound up in the molecule

²⁸ Jacques Loeb *Biochem Zeitschr*, **xix**, p 534, 1909

²⁹ See T Brailsford Robertson *Journ of Phys Chem*, **xiv**, p 528, 1910

Casein can be very accurately determined by making use of this reliable property and this method has been used in this investigation. The numbers representing the quantity of nitrogen, expressed in per cent, in purified casein from cow's milk obtained by different investigators are practically identical as may be observed from the following figures³⁰

Observer	Percentage of nitrogen in purified casein from cow's milk
Hammarsten	15.65
Chittenden and Painter	15.91
Lehmann and Hempel	15.60
Ellenberger	16.64
Average	15.70

From the mean of six determinations on the anhydrous purified casein used in the experiments described below I have obtained the number 15.81. To calculate the equivalent amount of casein from the nitrogen I have used the factor 6.4 which means that 1 cc $\frac{N}{10}$ alkali is equivalent to 9 mgs of casein. The method actually employed was as follows:

The casein was precipitated from 100 cc of the solutions under investigation by a slight excess of $\frac{N}{10}$ acetic acid (made up approximately by diluting 10 cc of Kahlbaum's glacial acetic acid to 1750 cc). The quantity of acetic acid varied from 15 to 30 cc according to the quantity of casein in solution. Not less than 15 cc was added even in the most dilute solutions of casein as a slight excess did not appear to affect the accuracy of the determination. Furthermore, the nature of the filtrates is an index to the quantity necessary for complete precipitation. The persistence of a cloudy filtrate after refiltering several times indicates incomplete precipitation which necessitates the addition of more acetic acid.

A cloudy filtrate which cannot be removed by repeated filtering very often results by directly precipitating casein in neutral or faintly alkaline solutions by acetic acid. This cloudiness can be overcome by redissolving the precipitate in a slight excess of alkali and immediately re-precipitating with acetic acid, or by adding just enough $\frac{N}{7}$ KOH (usually about 1 cc) to remove the opalescence in solutions of this type and then immediately adding acetic acid in slight excess to completely precipitate the casein. The latter method was adopted as it is more convenient and a clear filtrate always resulted when Schleicher and Schull's No. 590 "white band" filters were used. A finely divided precipitate which often occurs when casein is

³⁰ Quoted after Mann *Chemistry of the Proteids*, p. 397, MacMillan Co

precipitated in dilute solutions and especially in solutions of the hydroxides of the alkaline earths and which is difficult and often impossible to filter can be avoided if the acetic acid is added slowly (a few drops at a time) and the solutions vigorously shaken during precipitation and then allowed to stand for about an hour before filtering

The precipitate was thoroughly washed by decantation and on the filter with distilled water as free of CO_2 and NH_3 as could be achieved by boiling and the filter paper containing the precipitated casein transferred to a Kjeldahl digestion flask and the total nitrogen determined according to the Kjeldahl method ³¹

The following are some of the figures obtained by this method with purified anhydrous casein

SOLVENT	WEIGHED AMOUNT OF CASEIN IN 100 CC	ESTIMATED CASEIN
	mg	mg
NaOH	86	85
NaOH	238	235
NaOH	854	845
KOH	560	556
$\text{Ca}(\text{OH})_2$	324	319
$\text{Ca}(\text{OH})_2$	93	89
$\text{Ba}(\text{OH})_2$	738	728
$\text{Ba}(\text{OH})_2$	219	212

In obtaining the above figures it was necessary to work under conditions which would eliminate as far as possible the error due to hydrolysis. If the solution in which the casein is dissolved is too alkaline the high concentration of the OH ions causes rapid hydrolysis before the casein is completely dissolved. On the other hand, considerable amounts of casein will be hydrolyzed in very dilute solutions due to the long period of time which has elapsed before the state of complete solution is reached. Robertson³² observed this fact in his studies in the electrochemistry of the proteins while measuring the conductivity of solutions of potassium caseinate in solutions of varying OH ion concentrations, and it was found that the proportion, 10 cc of $\frac{N}{10}$ KOH to 1 gram of casein, gave the most satisfactory results.

³¹ The Kjeldahl method as described on page 5 of Bulletin No. 107 (Revised) of the Bureau of Chemistry, U. S. Department of Agriculture, was strictly followed in making the nitrogen determinations.

³² T. Brailsford Robertson *Journ. of Physical Chem.*, **14**, p. 528, 1910.

I have used this same proportion for NaOH and KOH but as casein dissolves more slowly in solutions of $\text{Ca}(\text{OH})_2$ and $\text{Ba}(\text{OH})_2$, a large proportion of alkali to casein (15 cc to 1 gram) was required. In preparing the casein solutions, therefore, from which the above results were obtained casein was dissolved in solutions of NaOH and KOH in the proportion of 10 cc of $\frac{N}{10}$ alkali to 1 gram of casein and in solutions of $\text{Ca}(\text{OH})_2$ and $\text{Ba}(\text{OH})_2$ in the proportion of 15 cc of $\frac{N}{10}$ alkali to 1 gram casein. Under these conditions hydrolysis would occur to a slight degree but I think the extent was reduced to a minimum. The casein was estimated in 100 cc of the respective solutions immediately upon complete solution by the method outlined above.

II EXPERIMENTAL

(a) General procedure

The casein employed in all of the experiments was Eimer and Amend's C P Casein "nach Hammarsten," further purified according to the method of Robertson³³ which is as follows

Half a pound of the casein was triturated with about 12 liters of distilled water, the water being added in six successive portions. On each addition of water the casein was well stirred up in it in a porcelain mortar and then allowed to settle, then the supernatant water was poured off and fresh water was added. It was then washed in a similar manner in 5 kilos of Kahlbaum's C P alcohol, 99.8 per cent, and then in 5 kilos of Kahlbaum's C P ether, distilled over sodium. The mortar containing the casein drained as free from superfluous ether as possible,³⁴ was then placed in an incubator over sulphuric acid at 40 to 50° C, the flame was turned out under the incubator and it was allowed to cool for about twenty-four hours. The casein is now found, if these operations have been conducted carefully, to be in the form of a dry, pure white powder, still containing, however, a considerable quantity of ether. The casein was now spread out, within the incubator, in a layer not over 1 cm deep, the flame under the incubator was lighted, fresh sulphuric acid was introduced if necessary, and it was allowed to stand for twenty-four hours at 40 to 50° C. The casein is then found to be free from appreciable water or ether.

³³ *Ibid*

³⁴ At this point it is necessary to avoid exposing the mortar to the moist air of the room a minute longer than is necessary, otherwise the evaporating ether causes condensation of sufficient moisture to spoil the product unless it is again treated with alcohol and ether.

Robertson finds that casein prepared in the above manner gives every indication of being a pure product. In one instance the same author³⁵ finds that casein thus prepared loses 5.8 per cent of its weight when dried for five hours at 70 to 80° C. I find that casein prepared as above loses 3.8 per cent of its weight after five hours' heating at 70° C or 4.19 per cent after five hours heating at 100° C. At 100° C, however, Lacqueur and Sackur³⁶ find that casein is decomposed.

The commercial trypsin prepared by Grüber of Leipzig was used in all of the experiments. This preparation contains a small insoluble residue and as more concordant results could be obtained with filtered solutions than with suspensions, filtered solutions were employed throughout, although they did not appear to be as active. Taylor³⁷ also observed the same conditions in his experiments on the hydrolysis of protamine by trypsin.

The incubator used was the double walled type employed by bacteriologists. It was provided with two doors, the inner a glass door and an outer double walled one. It would easily hold seventy-two Erlenmeyer flasks of 200 cc capacity, its inside dimensions being 36 cm deep, 45 cm wide, and 72 cm high. A temperature, constant within 0.5° C, could be maintained throughout the course of an experiment.

Schleicher and Schüll's "white band" quantitative filters No 590 (11 cm) were used throughout, as these were found to give an inappreciable blank in the nitrogen determinations and they held the precipitated casein especially well and filtration was comparatively rapid.

(b) *Relation between the time of hydrolysis and the amount of protein hydrolysed*

Henri and Larguer de Bancel's³⁸ have studied the digestion of gelatine and casein by trypsin using the electrical conductivity

³⁵ T Brailsford Robertson. *This Journal*, II, p 326, 1907.

³⁶ Lacqueur and Sackur. *Beiträge z chem Physiol und Pathol*, III, p 193, 1902.

³⁷ Taylor. On the Hydrolysis of Protamine with Especial Reference to the Action of Trypsin, *Univ Calif Pub Pathol*, I, p 7, 1904.

³⁸ Henri and de Bancel's. *Compt rend acad sci*, CXXXVI, pp 1038 and 1551, 1903.

method as measurement. They followed the curve only forty minutes, however, but during this brief interval it was found that the process of tryptic digestion follows the law for monomolecular reactions. The constants were also in fair agreement in series with two different substrate concentrations. Furthermore, their results confirm the hypothesis that the action of trypsin is not a pure catalytic reaction and that an intermediate compound is formed between the trypsin and substrate.

Bayliss³⁹ has investigated extensively the progress of the action of trypsin on casein and gelatine by the electrical conductivity method. He prepared an eight per cent solution of sodium caseinate and to 6 cc of this solution were added 2 cc of $\frac{N}{2}$ ammonia, 2 cc of a 2 per cent solution of trypsin, and a few drops of toluol. The conductivity was measured at different times and its increase at 39° C plotted in a curve which tends to become asymptotic to the base line, indicating that the velocity of the reaction approaches zero, and that an equilibrium point is reached before the reaction is completed. A mathematical analysis of his results shows that the velocity constant calculated from the monomolecular equation diminishes somewhat rapidly during the course of the reaction. The following are some of the values obtained when the equation $K = \frac{1}{t} \log_{10} \frac{a}{a-x}$, in which t is the time which has elapsed since the beginning of the reaction, a is the initial concentration of the substrate, and x is the amount of products formed during the time t , so that $a-x$ is the substrate concentration at the end of the time t , is applied to the rate of hydrolysis

First ten minutes	$K = 0.0079$
Second ten minutes	$K = 0.0046$
Third ten minutes	$K = 0.0032$
Fourth ten minutes	$K = 0.0022$
Fifth ten minutes	$K = 0.0016$
Seventh ten minutes	$K = 0.0009$
Ninth ten minutes	$K = 0.0007$

³⁹ Bayliss. The Kinetics of Tryptic Action, *Arch des sci biol*, 11 Suppl., p. 261, 1904, reprinted in the *Collected Papers of the Physiological Laboratory, University College, London*, viii

This phenomenon was due either to the retarding action of the products of hydrolysis or to the destruction of the trypsin or to both factors simultaneously. Bayliss worked with very alkaline solutions and this high degree of alkalinity must have caused a very rapid destruction of the trypsin.

In a very extensive investigation on the hydrolysis of casein by trypsin Robertson⁴⁰ by a different method found that the action of trypsin on calcium caseinate obeys the monomolecular formula during the first stages of the reaction. The amount of casein digested was estimated by a volumetric method based upon the fact that whenever a solution containing casein is neutral to phenolphthalein the proportion of base to casein = 80×10^{-5} equivalents per gram, i.e., using phenolphthalein as indicator, 1 gram of casein is almost exactly equivalent to 8 cc $\frac{N}{10}$ alkali solution. Briefly, the method consisted in dissolving the casein, which was precipitated by acetic acid, in a slight excess of a standardized $\text{Ca}(\text{OH})_2$ solution and subsequently titrating the uncombined alkali with a standard solution of HCl . From the results obtained the method appears to be a very accurate one. This method has been used by Hart⁴¹ in estimating the quantity of casein in milk.

With the view of throwing further light on this question, the following experiment was undertaken.

Seven liters of a 0.4 per cent solution of "basic" sodium caseinate were made by dissolving 28 grams of purified casein in 224 cc of $\frac{N}{10}$ NaOH and diluting to 7 liters with distilled water free from carbon dioxide and 100 cc placed in Erlenmeyer flasks of 200 cc capacity provided with tightly fitting rubber stoppers. Sixty-six flasks all of the same kind, lightly stoppered,⁴ were placed in the incubator and after the solution in each flask had reached the temperature of the incubator, as indicated by a thermometer immersed in the liquid, 0.2 cc toluol and 1 cc of a 0.2 per cent filtered solution of trypsin were added to each flask which was tightly closed and returned to the incubator and digested at $37.5^\circ \text{C} \pm 0.5^\circ$. Three samples to which no trypsin had been added were taken out and the casein determined in the usual way and is considered as the initial amount of casein present. Three samples were taken out and the casein determined in each after every fifteen

⁴⁰ T Brailsford Robertson. *This Journal*, 11, p 317, 1907.

⁴¹ Hart. *This Journal*, vi, p 445, 1909.

⁴ The flasks should not be closed tightly while being warmed, otherwise the increasing pressure may cause them to break.

minutes for the first three hours, after every half hour for the next three hours, and after every hour for the next three hours so that the reaction was followed for nine hours after 82.29 per cent of the casein had been completely hydrolyzed. To reduce the error of handling such a large number of samples to a minimum I proceeded in the following way. Toluol was first added to each flask after its contents had arrived at the temperature of the incubator, tightly closed, and replaced in the incubator. One sample at a time was then taken out, the trypsin solution added by means of a warmed pipette, and the time accurately noted. This sample was replaced in the incubator and allowed to digest for 9 hours. The second and third samples were withdrawn in like manner, the trypsin solution added, and the flask replaced and allowed to digest for nine hours. The next three samples treated exactly the same were allowed to digest for eight and one-half hours. This process was continued so that the last three samples were only allowed to digest for fifteen minutes. I repeated this operation three times on a small scale before the actual experiment was inaugurated and found that I could handle it very conveniently with the smallest possible error.

If this reaction obeys the law of mass action, the rate of change at any moment will be proportional to the concentration of the casein at that moment according to the equation

$$\frac{dx}{dt} = K(a - x) \quad (1)$$

where a is the initial amount of casein present, x the amount of it hydrolyzed in time t , and K the velocity constant. Integrating this expression, we get

$$-\ln(a - x) = Kt + \text{constant}$$

At the beginning of the reaction, $t = 0$, $x = 0$, and we have

$$-\ln a = \text{constant},$$

$$\ln a - \ln(a - x) = Kt$$

$$K = \frac{1}{t} \ln \frac{a}{a - x} \quad (2)$$

Now from the values of $a - x$ obtained it is possible to calculate the velocity constants at different times. Instead of using natural logarithms I have used common logarithms throughout which is 0.4343 times the natural. The following were the results obtained when equation (2) is applied to the rate of hydrolysis

TABLE I

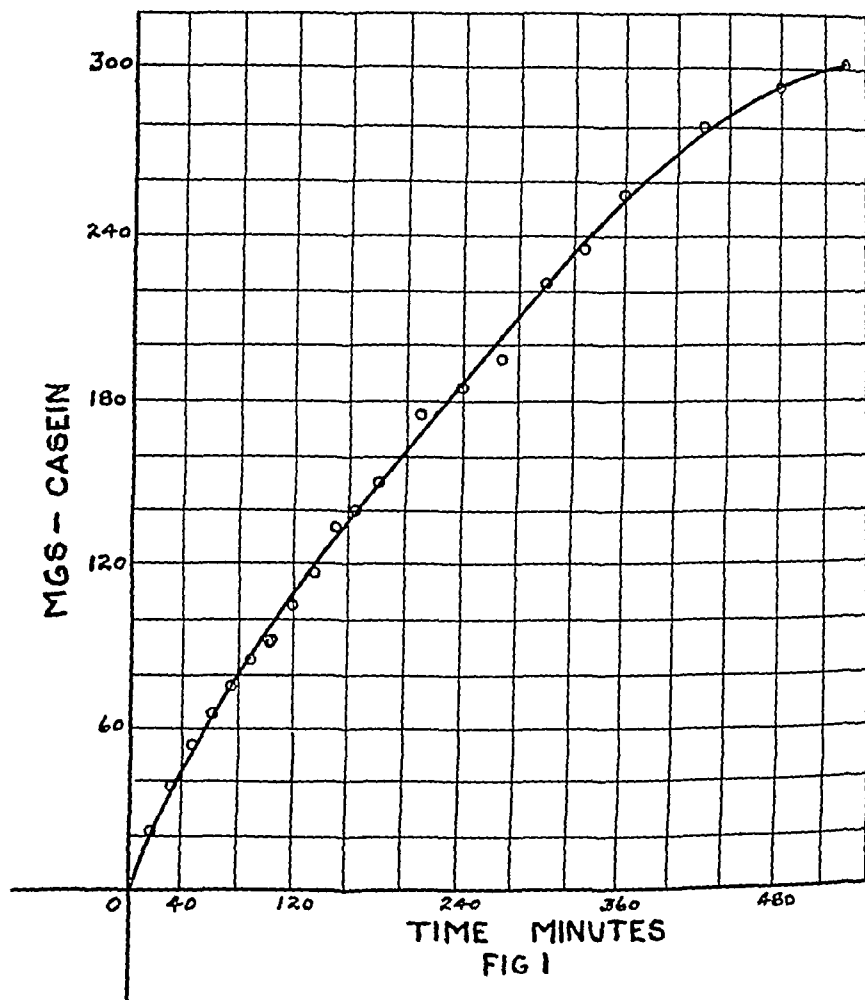
TIME IN MINUTES	CASEIN DIGESTED I.E., x	UNDIGESTED CASE- IN I.E. $a-x$	$\text{LOG IS } \frac{a}{a-x}$	K
	mg%	mg%		
0	0	367		
15	22	345	0 02685	18 $\times 10^{-4}$
30	38	329	0 04747	16 $\times 10^{-4}$
45	54*	313	0 06913	15 $\times 10^{-4}$
60	65	302	0 08466	14 $\times 10^{-4}$
75	75	292	0 09929	13 $\times 10^{-4}$
90	84	283	0 11288	12.5 $\times 10^{-4}$
105	94	273	0 12851	12 $\times 10^{-4}$
120	104	263	0 14471	12 $\times 10^{-4}$
135	115	252	0 16327	12 $\times 10^{-4}$
150	133	234	0 19545	13 $\times 10^{-4}$
165	142	225	0 21249	13 $\times 10^{-4}$
180	150	217	0 22821	12.5 $\times 10^{-4}$
210	174	193	0 27911	13 $\times 10^{-4}$
240	183	184	0 29985	12.5 $\times 10^{-4}$
270	194	173	0 32662	12 $\times 10^{-4}$
300	221	146	0 40032	13.5 $\times 10^{-4}$
330	234	133	0 44082	13 $\times 10^{-4}$
360	254	113	0 51159	14 $\times 10^{-4}$
420	278	89	0 61528	14.5 $\times 10^{-4}$
480	292	75	0 68961	14 $\times 10^{-4}$
540	302	65	0 75176	14 $\times 10^{-4}$

This number is the mean of two determinations

With one exception the figures in the second column are each the mean of three determinations. These results suffice to show very clearly that the velocity of hydrolysis at any moment is proportional to concentration of the casein at that moment. The slight lagging of the constant at the beginning of the experiment is in all probability due to some uncontrollable error in handling the experiment during such short intervals with the method employed and which would gradually be eliminated during the progress of the experiment. The results indicate, also, that the products of hydrolysis have very little, if any, influence upon the velocity of the reaction. Fig 1, in which the ordinates represent the amount of casein hydrolyzed in milligrams and the abscissae the time in minutes, shows the point in these experiments more lucidly.

- (c) *The relation between the concentration of the trypsin and the velocity of hydrolysis for different concentrations of protein*

The literature on the relation between ferment mass and the velocity with which a protein is hydrolyzed is strewn with many



contradictory statements. This confusion resulted largely from the use of the oft-described method of Mette⁴³ in determining the rate of hydrolysis. This method consists in subjecting short

⁴³ Mette quoted after Samojloff, *Arch des sci biol de St Petersburg*, II p 707, 1893

capillary glass tubes containing a solid protein such as fibrin or egg-albumin to the action of a ferment and measuring the quantity of protein dissolved. This method has been criticised by Taylor⁴⁴ who has shown that it possesses only a qualitative value for work of a physico-chemical nature. Moreover, the very limited knowledge concerning the nature and mode of action of ferments led to many questionable experimental conditions with respect to acidity, alkalinity, or salt content of the digests or the influence of various external factors which have recently been brought to light and shown to have a very considerable influence on the velocity with which a protein is hydrolyzed by an enzyme.

With the accumulation of facts pertinent to the chemical nature of the proteins, methods have sprung up whereby purer and much simpler substrates admitting of a more accurate measurement can be obtained. The more recent observers, therefore, concur in stating that the velocity of protein hydrolysis by an enzyme within a short range of temperature, and for certain concentrations of substrate, is directly proportional to the concentration of the ferment provided the latter is not rapidly destroyed by high concentrations of acids or alkalies.

As early as 1859 Brücke⁴⁵ studied the digestion of fibrin by pepsin and observed that the digestive power increased slowly as the quantity of pepsin was increased up to a certain limit above which additional quantities of pepsin had little or no effect. This early observation by Brücke was confirmed by the subsequent investigation of Maly,⁴⁶ Mayer,⁴⁷ Ellenberger and Hofmeister⁴⁸ and Klug.⁴⁹

Borissoff⁵⁰ in 1891 by the aid of Mette's method, found that

⁴⁴ Taylor. On the Hydrolysis of Protamine with Especial Reference to the Action of Trypsin, *Univ of Calif Pub Pathol*, 1, p 7, 1904

⁴⁵ Brücke. *Sitzungsber Wien Acad*, xxxvii, p 131, 1859, quoted after Samojloff *Arch des sci biol de St Petersburg*, ii, p 701, 1893

⁴⁶ Maly. Hermann's *Handbuch der Physik*, v, (2) p 73, 1881

⁴⁷ Mayer. *Zeitschr f Biol*, xvii, p 351, 1881

⁴⁸ Ellenberger and Hofmeister. *Arch f Wiss u pract Therheil* ix, p 185 1883, quoted after Carl Oppenheimer, *Ferments and their Action*, Eng trans by Mitchell, London, 1901, p 95

⁴⁹ Klug. *Pflüger's Archiv*, lv, p 43, 1895

⁵⁰ Borissoff. *La substance zymogene de la pepsine et sa transformation en pepsine active*, (Thesis in Russian) St Petersburg, 1891, quoted after Samojloff, *Arch des sci biol de St Petersburg* ii, p 705, 1893

the rate of digestion of egg-albumin by trypsin was proportional to the square root of the quantity of trypsin present, and Samojloff⁵¹ using the same method found the same rule to hold good only in dilute solutions. E. Schutz⁵² showed that the velocity of the hydrolysis of fibrin by pepsin was proportional to the square root of the quantity of the ferment when the quantity varied as 1 to 64, and Jul. Schutz⁵³ finds that this rule holds for the peptic digestion of egg-albumin, and Walter⁵⁴ and Vernon⁵⁵ confirm it for digestion by trypsin.

In 1895 Sjöqvist⁵⁶ introduced a new method for determining the velocity of hydrolysis of proteins by ferments. He noted the changes in the electrical conductivity in solutions of egg-albumin when acted upon by pepsin and hydrochloric acid. In his experiments four different concentrations of pepsin were allowed to act upon constant concentrations of egg-albumin at 37° C and the conductivity of the different solutions measured at given intervals. He found that the rate of hydrolysis during the first stages of the reaction was proportional to the square root of the mass of pepsin.

On the other hand Schutz and Huppert,⁵⁷ Pollok,⁵⁸ and Sawjalow⁵⁹ by the aid of Mette's method were unable to confirm the rule of Schutz but found that the quantity of digested protein was proportional to the quantity of ferment. Loehlein⁶⁰ found the rate of hydrolysis proportional to the square root of the quantity of pepsin and directly proportional for trypsin. He employed the acidimetric method of Volhard⁶¹ which consists in precipitating the undigested casein by sodium sulphate from solutions of

⁵¹ Samojloff *Arch des sci biol de St Petersburg*, II, p 699, 1893

⁵² Emil Schutz *Zeitschr f physiol Chem*, IX, p 577, 1895

⁵³ Julius Schutz *Zeitschr f physiol Chem*, XX, p 1, 1900

⁵⁴ Walter *Arch des sci biol de St Petersburg*, VII, p 1, 1899

⁵⁵ Vernon *Journ of Physiol*, XXVI, p 405, 1900

⁵⁶ John Sjöqvist *Skand Arch f Physiol*, V, (part III), p 354, 1895

⁵⁷ E. Schutz and Huppert *Pflüger's Archiv*, LXX, p 470, 1900

⁵⁸ Pollok *Beitr z chem Physiol u Pathol*, VI, p 95, 1904

⁵⁹ Sawjalow *Zeitschr f physiol Chem*, XLVI, p 307, 1905

⁶⁰ Loehlein *Beitr z chem Physiol u Pathol*, VII, p 120, quoted after Taylor, *Univ Calif Pub Pathol*, I, p 243, 1907

⁶¹ Volhard *Münch med Wochenschr*, Nos 49 and 50, 1903, quoted after T. Brailsford Robertson, *This Journal*, II, p 328, 1907

casein hydrochloride, and subsequently titrating the quantity of free acid in the filtrate

Faubel⁶² by the aid of the same method found that digestion by trypsin was proportional to the concentration of the ferment Fuld⁶³ and Gross⁶⁴ found that the time required for digestion by trypsin is inversely proportional to the quantity of ferment Gross followed the reaction by noting the moment at which the digesting solution ceased to give a precipitate with 1 per cent acetic acid Palladin⁶⁵ has recently studied digestion by trypsin and found that when a protein is in a state of solution the amount hydrolyzed is directly proportional to the concentration of the ferment The author made use of the following method A solid protein (fibrin) was dyed with "spirit blue, blue shade" and immersed in a solution of trypsin It was found that as hydrolysis proceeds the solution becomes colored proportionately to the amount of protein dissolved By making colorimetric comparisons with solutions of known concentrations the amount of protein digested was estimated The method was also employed to determine the quantity of trypsin in solutions of unknown concentrations

Weis⁶⁶ carried out some experiments on the digestion of the protein from wheat by means of malt extract In some of his experiments the concentration of protein was varied as well as the concentration of the ferment (trypsin or pepsin) It was observed that within a certain limit the amount of protein digested was proportional to the quantity of acting ferment above which it varied as the concentration of the substrate Arrhenius⁶⁷ and Euler⁶⁸ have calculated the constants from the results obtained by Weis and Arrhenius concludes that the quantity of protein hydrolyzed is inversely proportional to the square root of the substrate concentration The somewhat complicated sys-

⁶² Faubel *Beitr zur chem Physiol u Pathol*, x, p 35, 1907

⁶³ Fuld *Arch f exp Pathol u Pharm*, lviii, p 468, 1908

⁶⁴ Gross *Arch f exp Pathol u Pharm*, lviii, p 157, 1908

⁶⁵ Palladin *Pfluger's Archiv*, cxxxv, p 337, 1910

⁶⁶ Weis *Compt rend des trav du lab d Carlsberg*, v, p 133, 1900-03

⁶⁷ Arrhenius *Immunochemistry*, p 84, MacMillan Co, New York, 1907

⁶⁸ Euler *Allgemeine Chemie der Enzyme*, p 130, Wiesbaden, Verlag von J F Bergmann

tem (substrate and ferment) used in these experiments renders the results not very convincing

Henri and Larguier des Bancelles (*loc cit*) and Bayliss (*loc cit*) by the electrical conductivity method confirmed the rule of direct proportionality for the tryptic digestion of gelatin and casein, and Taylor (*loc cit*) showed that the tryptic digestion of protamine obeys the same law

Hedin⁶⁹ studied the digestion of casein, serum-albumin, and white of egg by trypsin. He found that the rate of digestion was directly proportional to the quantity of ferment acting when all other conditions were kept constant, and also the rate of digestion is directly proportional to the time under otherwise constant conditions. In other words, the velocity of hydrolysis obeys the formula, $\log_{10} \frac{a}{a-x} = Kft$, where a is the initial concentration of substrate, x the amount of it transformed in time t , f the concentration of ferment, and K the velocity constant. A direct proportionality between the substrate concentration and the rate of change was observed, although the constants were not concordant for different substrate concentrations and the effect per unit of casein increases as the total amount of casein diminishes, and finally becomes constant. The activity of the ferment diminished during the course of the reaction and the products of hydrolysis appeared to exert a considerable depressant influence. It was also noted that the total effect was not affected by dilution with water, in other words, if the ratio between the ferment and trypsin be kept constant, the effect for equal volumes is proportional to the concentration. Hedin measured the reaction by estimating the quantity of nitrogen that escaped precipitation by tannic acid. This method has been criticised by Taylor⁷⁰. Later experiments on the digestion of casein by trypsin yielded similar results when the rate of change was measured by estimating the quantity of phosphorus split off during the course of the reaction.

Quite recently Robertson⁷¹ made an investigation of this subject using a more refined method of measurement. Calcium

⁶⁹ Hedin *Journ of Physiol*, xxxii, p 468, 1904, xxxiv, p 370, 1906

⁷⁰ Taylor *On Fermentation, Univ Calif Pub Pathol*, i, p 236, 1907

⁷¹ T Brailsford Robertson *This Journal*, ii, p 317, 1907

and barium caseinates were used as substrates. It was found that the velocity of hydrolysis of calcium caseinate is directly proportional to the amount of trypsin for small concentrations of substrate. For concentrations, above $\frac{N}{400}$ Ca(OH)_2 saturated with casein, the velocity constant calculated from the monomolecular formula increased with increasing quantities of ferment. In this case the author states that the constant for the the velocity of hydrolysis is equal to $af + bf^2$, where f is the concentration of the ferment and a and b are constants. By representing the ratio $\log_{10} \frac{a}{a-x}$ to the number of cubic centimeters of trypsin solution by y it was found that $y = 41 + 11f$. This phenomenon was not improbably due to the destruction of the ferment by the uncertain OH^- concentrations of the solutions employed as my observations are not in accord with this theory.

In most of the experiments quoted above the concentrations of the substrate were almost always constant. It appeared important, therefore, to repeat some of these experiments with various concentrations of protein. Basic sodium caseinate was used as substrate in all of the experiments and observations were made on the relation between the concentration of the trypsin for 0.125, 0.25, 0.5, 1, 2, and 4 per cent solutions.

EXPERIMENT 1 Eight grams of purified casein were dissolved in 64 cc $\frac{N}{10}$ NaOH and diluted to 6400 cc with distilled water free from carbon dioxide. Sixty-two Erlenmeyer flasks of 200 cc capacity containing 100 cc of the above solution were lightly stoppered with clean rubber stoppers and placed in the incubator. As soon as the solutions had reached the temperature of the incubator, 0.2 cc of toluol were added to each digest and the flasks tightly stoppered and replaced in the incubator. After the temperature of the solutions was readjusted, two samples were removed and the casein determined in each which gave the initial amount of casein present. As quickly as possible 0.5 cc of a 0.025 per cent solution of trypsin were added to 6 flasks (set A), one flask at a time being removed from the incubating oven, and allowed to digest at $37.5^\circ \text{C} \pm 0.25^\circ$. To six flasks of another set (B) was added 1 cc of trypsin solution, to six flasks of another set (C) 2 cc, to six flasks of another set (D) 3 cc and so forth up to 9 cc of trypsin solution, thus making ten sets of six flasks each representing ten different concentrations of ferment to a constant concentration of substrate. Two flasks from each set were withdrawn after one, two, and three hours and the undigested casein determined in each in the usual way. In sets A, B, and C

containing 0.5, 1, and 2 cc of trypsin respectively, the casein digested during the first two hours was too slight to be measured accurately and so the tabulated results for these sets give only the change that occurred during the first three hours

Now if the velocity of transformation is directly proportional to the concentration of the trypsin, formula (2) becomes

$$\text{Log}_{10} \frac{a}{a-x} = K_1 f t \quad (3)$$

where K_1 is a constant and f is the concentration of the ferment. The following tables contain the observed results as well as those calculated by means of the above formula. The initial amount of casein in each digest was found to be 108 mgs

TABLE II

Series A, B, and C

$a = 108 \quad t = 3 \text{ hours}$

f CC TRYPSIN	$a - x$	$\text{LOG}_{10} \frac{a}{a-x}$	λ_1
0.5	104.85	0.01285	8.5×10^{-3}
1	98.70	0.03910	13×10^{-3}
2	90.90	0.07486	12.5×10^{-3}

Series D

$f = 3 \text{ cc}$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a-x}$	λ_1
0	108		
1	100.44	0.02974	10×10^{-3}
2	91.62	0.07143	12×10^{-3}
3	87.93	0.08928	10×10^{-3}

Series E

$f = 4 \text{ cc}$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a-x}$	λ_1
0	108		
1	98.10	0.04175	10.5×10^{-3}
3	83.50	0.11173	9.5×10^{-3}

TABLE II—Continued

Series F

$$f = 5\text{cc}$$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
0	108		
1	97 20*	0 04575	9×10^{-3}
2	85 28	0 10257	10×10^{-3}
3	78 63	0 13783	9×10^{-3}

This number is based upon one determination

Series G

$$f = 6\text{cc}$$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
0	108		
1	93 15	0 06424	10.5×10^{-3}
2	80 43	0 12800	10.5×10^{-3}
3	72 90	0 17069	9.5×10^{-3}

Series H

$$f = 7\text{cc}$$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
0	108		
1	88 43	0 08682	12.5×10^{-3}
2	79 29	0 13420	9.5×10^{-3}
3	68 04	0 21066	10×10^{-3}

Series I

$$f = 8\text{cc}$$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
0	108		
1	87 53	0 09126	11.5×10^{-3}
2	75 6	0 15438	9.5×10^{-3}
3	64 53	0 22366	9.5×10^{-3}

TABLE II—Continued

Series J

 $f = 9\text{cc}$

t HOURS	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
0	108		
1	85 95	0 09917	11×10^{-3}
2	73 80	0 16536	9×10^{-3}
3	60 75	0 24987	9×10^{-3}

The numbers in the column under $a - x$ are each the mean of two determinations. The figures in the last column are very satisfying and show that at this substrate concentration the velocity of hydrolysis is proportional to the concentration of the ferment. The figure on the following page brings out this fact more clearly.

EXPERIMENT 2 Four grams of purified casein were dissolved in 32 cc $\frac{N}{10}$ NaOH and diluted to 1600 cc with distilled water free from carbon dioxide, and 100 cc measured out in each of 12 Erlenmeyer flasks of 200 cc capacity and 0.2 cc toluol and 1, 3, 5, 7, and 9 cc of a 0.065 per cent solution of trypsin was added to the digests. The experiments were carried out in duplicate. The initial quantity of casein was found to be 217.35 mgs, this being the mean of two determinations. The solutions were allowed to digest at $38.5^\circ \text{C} \pm 0.5^\circ \text{C}$ for three hours. The results obtained are tabulated in the following table.

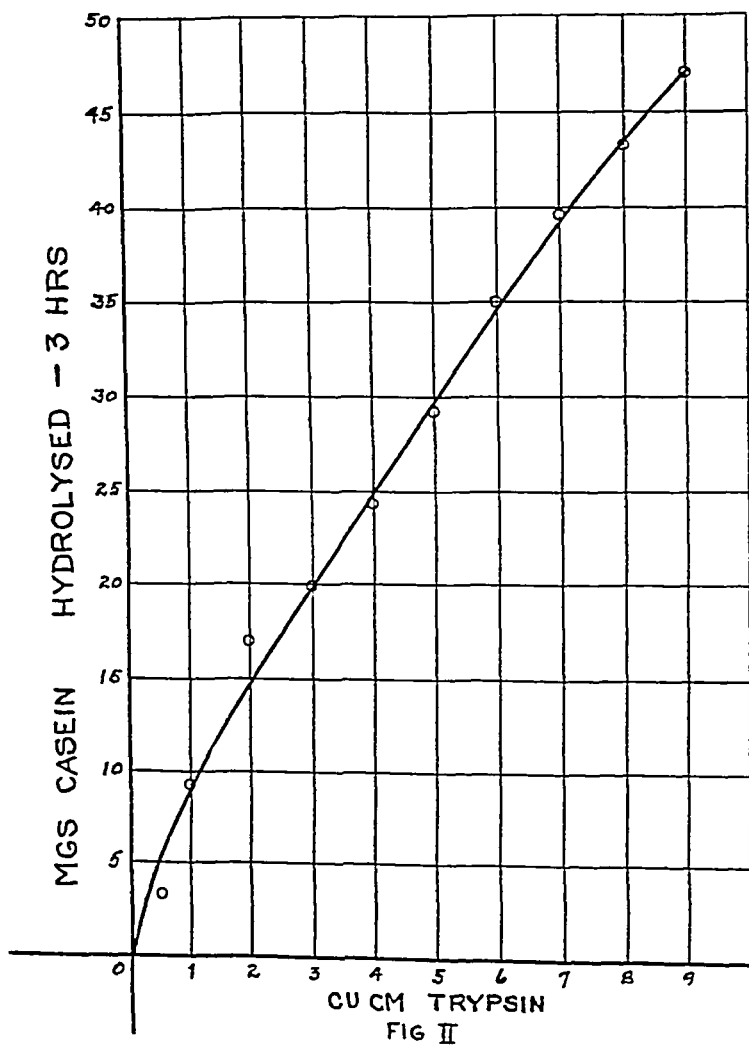
TABLE III

 $a = 217.35$ $t = 3$ hours

f CUBIC CENTIMETERS TRYPSIN	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
1	170 10	0 10646	35.5×10^{-3}
3	103 05	0 31165	34.5×10^{-3}
5	60 00	0 53782	36×10^{-3}
7	37 35	0 76487	36.5×10^{-3}
9	26 10	0 92052	34×10^{-3}

The figures in the second column are each the mean of two determinations. The figures in the last column are again fairly constant.

EXPERIMENT 3 The experimental procedure was exactly as in the last experiment except that a 0.5 per cent solution of "basic" sodium caseinate was used instead of a 0.25 per cent solution. Eight grams of casein were dissolved in 64 cc $\frac{N}{10}$ NaOH and diluted to 1600 cc. The quantities of trypsin solution used were 2, 4, 6, 8, and 10 cc of a 0.1 per cent solution. The



quantity of casein initially in 100 cc was found to be 428.4 milligrams. The following are the experimental results. The digestion was carried out at $38.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for three hours.

TABLE IV
 $a = 428.4 \quad t = 3 \text{ hours}$

f CUBIC CENTIMETERS TRYPsin	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
2	294.3	0.16306	27×10^{-3}
4	184.5	0.36585	30.5×10^{-3}
6	101.7	0.62453	34.5×10^{-3}
8	60.3	0.85153	35.5×10^{-3}
10	40.05	1.02925	34.5×10^{-3}

The experiment was done in duplicate and the figures in the second column are each the mean of two determinations.

EXPERIMENT 4 Exactly as in the two previous experiments except that a 1 per cent solution of casein, 16 grams of pure casein dissolved in 128 cc $\frac{N}{10}$ NaOH and diluted to 1600 cc, was used in place of a 0.5 per cent solution. The concentrations of the trypsin used were 1, 2, 4, 6, and 8 cc of a 0.2 per cent solution. The digestion was carried out at $39.5^{\circ}\text{C} \pm 0.5^{\circ}$ for three hours.

TABLE V
 $a = 857.8 \quad t = 3 \text{ hours}$

f CUBIC CENTIMETERS TRYPsin	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
1	651.75	0.11931	40×10^{-3}
2	486.54	0.24628	41×10^{-3}
4	293.90	0.46519	39×10^{-3}
6	161.23	0.72594	40×10^{-3}
8	90.12	0.97857	41×10^{-3}

EXPERIMENT 5 In this experiment a 2 per cent solution of "basic" sodium caseinate, made by dissolving 32 grams of casein in 256 cc $\frac{N}{10}$ NaOH and diluting to 1600 cc, was used as substrate. 1, 2, 4, 6, and 8 cc of a 0.4 per cent solution of trypsin were employed. The digestion was carried out for three hours at $39^{\circ}\text{C} \pm 0.5^{\circ}$. The initial quantity of casein in 100 cc was found to be 1618 mgs.

TABLE VI
 $a = 1618 \quad t = 3 \text{ hours}$

f CUBIC CENTIMETERS TRYPSIN	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
1	1239	0 11591	38.5×10^{-3}
2	965	0 22400	37.5×10^{-3}
4	573	0 45083	37.5×10^{-3}
6	332	0 68784	38×10^{-3}
8	223	0 86068	36×10^{-3}

EXPERIMENT 6 Precisely as in the previous experiments except that a 4 per cent solution of "basic sodium" caseinate, made by dissolving 64 grams of purified casein in 512 cc $\frac{N}{10}$ NaOH and diluting to 1600 cc, was employed as substrate. The concentrations of trypsin used were 1, 3, 5, 7, and 9 cc of a 0.8 per cent solution. The initial quantity of casein in 100 cc was found to be 3123 mgs. The solutions were allowed to digest for three hours at $39^\circ \text{C} \pm 0.5^\circ$. The following results were obtained.

 TABLE VII
 $a = 3123 \quad t = 3 \text{ hours}$

f CUBIC CENTIMETERS TRYPSIN	$a - x$	$\text{LOG}_{10} \frac{a}{a - x}$	K_1
1	2420	0 11075	37×10^{-3}
3	1437	0 33711	37.5×10^{-3}
5	846	0 56720	38×10^{-3}
7	544	0 75897	36×10^{-3}
9	407	0 88498	33×10^{-3}

In the foregoing experiments it will be observed that the proportion of ferment to protein was practically the same and that the velocity constants in the entire series approach the same value, the non-concordance being due to the decay of the ferment through physical disturbances. It is a well known fact that trypsin preparations gradually lose their digestive power by the physical disturbances of the laboratory.⁷²

EXPERIMENT 7 In this experiment five different concentrations of "basic" sodium caseinate (0.2, 0.4, 1, 2, and 4 per cent solutions) were

⁷² See Taylor. On the Hydrolysis of Protamine with Especial Reference to the action of Trypsin, *Univ Calif Pub Pathol*, 1, p 7, *ibid*, On Fermentation, p 249, 1907.

digested simultaneously with varying amounts of the same ferment solution. A 0.2 per cent solution of trypsin was used throughout and the digestion was carried out for three hours at $36^{\circ}\text{C} \pm 0.5^{\circ}$. The following results were obtained. The figures in the column under $a - x$ are each the mean of two determinations.

TABLE VIII

(A)

 $a = 177 \text{ mgs}$

TRYPSIN f	CASEIN DIGESTED IN THREE HOURS x	UNDIGESTED CASEIN $a - x$	$\frac{L}{f} \log_{10} \frac{a}{a - x}$	$k \times 10^3$
cc	mgs	mgs		
1	119	58	0.161513	28.5
2	156	21	0.154291	27.5
Average				28

(B)

 $a = 360 \text{ mgs}$

1	144	216	0.0739500	26.5
2	233	127	0.0754160	27.0
3	280	80	0.0725786	26.0
4	324	46	0.0744616	27.0
Average				26.6

(C)

 $a = 882 \text{ mgs}$

1	149	733	0.026790	23.5
2	262	620	0.025513	22.5
3	380	502	0.027196	24.0
4	468	414	0.027373	24.0
Average				23.5

(D)

 $a = 1733 \text{ mgs}$

2	337	1396	0.015651	27
3	463	1270	0.015000	26
4	528	1205	0.013151	23
10	1055	678	0.013585	23.5
Average				24.9

TABLE VIII—Continued

(E) $a = 3326$ mgs				
2	320	3006	0 007322	24 5
4	518	2808	0 006126	20 5
6	750	2576	0 006165	20 5
10	1136	2190	0 006049	20 0
Average				21 4

The results of the above experiments show quite conclusively that the velocity with which casein is hydrolyzed by trypsin is directly proportional to the concentration of the ferment. They also indicate a general proportionality between the velocity of hydrolysis and the concentration of the substrate. The slight variations that occur in the constants in the different experiments are due to slight differences in temperature and to small variations in the intensity of the ferment. The last experiment brings out the notable fact that the constants decrease as the concentration of the substrate increases indicating a slight tendency to depart from the rule of direct proportionality. This fall in the velocity constant however is not so great in my experiments as was observed by Taylor⁷³ in the tryptic digestion of protamine. In fact it was so slight that it escaped unobserved in the previous experiments. It is a fact to be noted that by increasing the concentration of the ferment the turbidity of the casein solutions is increased. This fact is striking particularly in the case of strong solutions, from 0.5 to 4 per cent. The turbidity of these solutions gradually increased by the addition of increasing quantities of ferment so that the solution containing the greatest amount of trypsin (10 cc) resembled a solution of calcium caseinate of the same concentration which is, normally, very much more turbid than solutions of sodium caseinate.

(d) *The relation between the nature of the base combined with a protein and the velocity with which it is hydrolyzed*

There has been little said concerning the relation between the nature of the base combined with a protein and the velocity

⁷³ Taylor. On Fermentation, *Univ Calif Pub Pathol*, 1, p 236, 1907

with which it is hydrolyzed Robertson (*loc cit*) made some experiments using calcium and barium caseinates as substrates and found that the velocity constant for the hydrolysis of barium caseinate is only about two-thirds as great as for calcium caseinate. These experiments, however, were not carried out simultaneously and this difference may possibly be due to differences in the digestive power of the ferments used. Also, there may possibly have been a slight difference in the degree of alkalinity of the two solutions employed as the solutions were made by shaking up solutions of $\frac{N}{600}$ alkali with casein. A later investigation revealed the fact that $\text{Ca}(\text{OH})_2$ dissolves casein more rapidly than $\text{Ba}(\text{OH})_2$,⁷⁴ and also that casein combines with bases in equivalent molecular proportions.⁷⁵

From a consideration of the rôle of inorganic substances in nutrition as well as for theoretical reasons, it appeared of some import to carry out some experiments with the various caseinates of the alkalies and alkaline earths. Experiments were made with 0.4 and 2 per cent solutions of basic Li, Na, K, NH_3 , Ca, Sr, and Ba caseinates. Solutions were made in the usual way so that the proportion of base to casein = 80×10^{-5} equivalents per gram. The experimental procedure was precisely the same as in those described above and the experiments were done in duplicate and simultaneously for each substrate concentration so that the acting mass and intensity of the ferment would be the same in each series. In series A of 0.4 per cent solutions of casein, 1 cc. of a 0.5 per cent solution of trypsin and 0.2 cc. of toluol were added to 100 cc. of the hydrolyte and the digestion carried out at $37^\circ \text{C} \pm 0.5^\circ$ for three hours. To each digest of 100 cc. of the 2 per cent solutions in series B were added 1 cc. of a 3 per cent solution of trypsin and 0.2 cc. toluol and the digestion allowed to continue at $38^\circ \text{C} \pm 0.5^\circ$ for three hours. The following results were obtained

⁷⁴ T. Brailsford Robertson, *Journ. of Physical Chem.*, xiv, p. 377, 1910.

⁷⁵ T. Brailsford Robertson, *Ibid.*, xv, p. 179, 1911.

SERIES A, 0.4 PER CENT SOLUTIONS

(Temperature $37^{\circ} \pm 0.5^{\circ}$)

TABLE IX

0.4 per cent basic lithium caseinate

HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	K
0	377		
1	232	0.21085	21×10^{-2}
2	125	0.47943	24×10^{-2}
3	73	0.71302	24×10^{-2}

TABLE X

0.4 per cent basic sodium caseinate

t HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	K
0	367		
1	225	0.21249	21×10^{-2}
2	133	0.44082	22×10^{-2}
3	68	0.73216	24×10^{-2}

TABLE XI

0.4 per cent basic potassium caseinate

t HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	K
0	377		
1	222	0.22999	23×10^{-2}
2	139	0.43333	22×10^{-2}
3	80	0.67325	$22 = 10^{-2}$

TABLE XII

0.4 per cent basic ammonium caseinate

t HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	K
0	376		
1	230	0.21346	21.5×10^{-2}
2	128	0.46798	23.5×10^{-2}
3	69	0.73684	24.5×10^{-2}

TABLE XIII

0.4 per cent basic calcium caseinate

<i>t</i> HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	352		
1	220	0.20412	20×10^{-2}
2	139	0.40353	20×10^{-2}
3	69	0.70769	23.5×10^{-2}

TABLE XIV

0.4 per cent basic strontium caseinate

<i>t</i> HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	351		
1	222	0.19896	20×10^{-2}
2	130	0.43137	21.5×10^{-2}
3	67	0.71924	24×10^{-2}

TABLE XV

0.4 per cent basic barium caseinate

<i>t</i> HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	357		
1	222	0.20632	20.5×10^{-2}
2	144	0.39431	20×10^{-2}
3	75	0.67761	22.5×10^{-2}

SERIES B, 2 PER CENT SOLUTION

(Temperature $38^{\circ} \pm 0.5^{\circ}$)

TABLE XVI

2 per cent basic lithium caseinate

<i>t</i> HOURS	$a-x$ MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1706		
1	930	0.26350	26×10^{-2}
2	558	0.48535	24×10^{-2}
3	302	0.75197	25×10^{-2}

TABLE XVII

2 per cent basic sodium caseinate

<i>t</i> HOURS	<i>a</i> - <i>x</i> MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1679		
1	909	0 26649	25.6×10^{-2}
2	563	0 47454	24×10^{-2}
3	294	0 75670	25×10^{-2}

TABLE XVIII

2 per cent basic potassium caseinate

<i>t</i> HOURS	<i>a</i> - <i>x</i> MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1665		
1	896	0 26909	27×10^{-2}
2	509	0 51074	25.5×10^{-2}
3	289	0 75658	25×10^{-2}

TABLE XIX

2 per cent basic ammonium caseinate

<i>t</i> HOURS	<i>a</i> - <i>x</i> MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1662		
1	887	0 27271	27×10^{-2}
2	540	0 48824	24.5×10^{-2}
3	297	0 74787	25×10^{-2}

TABLE XX

2 per cent basic calcium caseinate

<i>t</i> HOURS	<i>a</i> - <i>x</i> MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1728		
1	990	0 27190	24×10^{-2}
2	660	0 41800	21×10^{-2}
3	358	0 68366	23×10^{-2}

TABLE XXI
2 per cent basic strontium caseinate

<i>t</i> HOURS	<i>a - x</i> MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1670		
1	950	0 24500	24.5×10^{-2}
2	600	0 44457	22×10^{-2}
3	340	0 69124	23×10^{-2}

TABLE XXII
2 per cent basic barium caseinate

<i>t</i> HOURS	<i>a - x</i> MILLIGRAMS	$\text{LOG}_{10} \frac{a}{a-x}$	<i>K</i>
0	1701		
1	980	0 23947	24×10^{-2}
2	650	0 41779	21×10^{-2}
3	350	0 68663	23×10^{-2}

From an examination of the above tables it will be noticed that the constants are quite concordant, especially in the weaker solutions. For high substrate concentrations, however, there is a slight tendency downwards in the case of the salts of the alkaline earths which are hydrolyzed with a slightly lower velocity than the corresponding salts of the alkalis. It is concluded that the nature of the base combined with a protein has little or no influence in the process of hydrolysis.

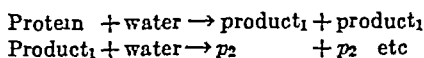
It is also interesting to note in this connection that the velocity of hydrolysis is uninfluenced by the extent to which the "basic" caseinates of the alkalis and alkaline earths are dissociated into their respective ions. Robertson⁷⁶ has calculated the dissociation-constants of the "basic" caseinates of the alkalis and alkaline earths on the assumption that the "basic" caseinates dissociate into two protein ions each possessed of twice as many valencies as there are molecules of base bound up in the molecule of the caseinate. It was found that at 0.01 N concentration (0.005 N concentration of the neutralized alkali or 0.01 N concentration of the neutralized alkaline earth) the caseinates of the alkalis are almost completely dissociated while the caseinates of the alkaline earths are only about 50 per cent dissociated.

⁷⁶ T. Brailsford Robertson *Ibid.*, xiv, p. 60, 1910.

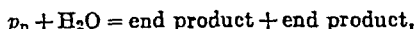
III DISCUSSION AND RÉSUMÉ OF RESULTS

The mode of action of trypsin in the above experiments corresponds to case II outlined by Armstrong⁷⁷ in explaining the action of sucroclastic enzymes. This is a case in which the concentration of the ferment is relatively large and practically unaffected by the products of change. The active mass is a function of the total mass from the beginning of the experiment and the change is expressed as a logarithmic function of the time.

Now as Taylor⁷⁸ has pointed out, the fermentation of a protein belongs to a class of mediated catalysis in which the transformation proceeds in many stages according to the following



and finally



each stage never being completed *en bloc*. As we have seen the process of hydrolysis obeys the monomolecular formula, which demands as a first postulate that the reacting substance exists at each moment in the form of unchanged substrate or end product. We must conclude, therefore, that we only follow the course of the first reaction and whatever the velocities or modes of transformation of the intermediary reactions are they do not perceptibly alter the course of the reaction measured. The well known monomolecular formula does not anticipate a reversion of the reaction and according to the foregoing experiments there is no indication of such. The reaction proceeds according to the law to the point of equilibrium which appears to be near the point of complete hydrolysis.

The synthesis of proteins by a reversion of the above reaction through the action of enzymes is now a matter of fact. Taylor⁷⁹ was able to effect the synthesis of protamine through the action of trypsin and Robertson⁸⁰ has synthesized paranuclein through

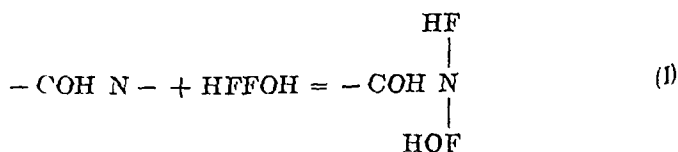
⁷⁷ Armstrong *Proc of the Royal Soc of London*, lxxiii, p 500, 1904.

⁷⁸ Taylor *On Fermentation*, *Univ Calif Pub Pathol*, 1, p 125, 1907.

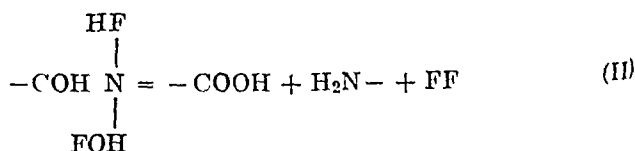
⁷⁹ Taylor *On the Synthesis of Protein through the action of Trypsin*, *Univ Calif Pub Pathol*, 1, p 343, 1907. *This Journal*, iii, p 87, 1907.

⁸⁰ T Brailsford Robertson *This Journal*, v, p 493, 1908, *Ibid*, vi, p 95, 1907, *Univ Calif Pub Physiol*, iii, p 59, 1907.

the agency of pepsin. In these experiments the protein synthesis was brought about by allowing large quantities of the respective ferments to act upon concentrated solutions of the products of the complete digestion of the respective proteins. These experiments suffice to show the action of ferments in accelerating the reverse of the reaction alluded to above. Now if there is no indication of reversion in the process of hydrolysis how is this phenomenon to be explained? For the interpretation of this Robertson⁸¹ has advanced an hypothesis of *Reciprocal Catalysis* which appears to be a rational explanation of the above facts. This hypothesis assumes a combination between the enzyme and protein,⁸² the enzyme being assumed to carry water into the protein molecule and parting with the water to recoup itself from the medium, while the protein molecule subsequently splits up into the products of its hydrolysis, and the enzyme-product finally reacting with the water regenerating the ferment. Considering both the protein and enzyme to be amphoteric electrolytes, which is a reasonable conclusion from experimental data, the various steps in the process of hydrolysis are represented by the following schematic equations



Protein + Ferment = Protein-ferment compound



Protein-ferment compound = Product + Product + Anhydrous Ferment



Anhydrous Ferment + Water = Hydrated Ferment

⁸¹ T Brailsford Robertson *loc cit*, *Die physikalische Chemie der Proteine*, Dresden, Verlag von Theodor Steinkopff, p 404

⁸² Biological chemists have pretty generally accepted this to be a fact, see literature quoted elsewhere in this paper under Vernon, Bayliss, Robertson, Henri, and Hedin

while the synthesis is the reverse of these reactions, thus assuming the existence of two varieties of the same ferment, one accelerating hydrolysis, and the other accelerating synthesis, each operating under very different conditions. The existence of two such forms of an enzyme has been observed by Euler.⁸³

It is supposed that during the process of hydrolysis the station of equilibrium in reaction III is far to the right and is reached with great velocity compared with that of either of the reactions I and II measured in the direction from right to left and at any moment the concentration of the anhydrous (synthesis-accelerating) form of the ferment FF is negligible compared with that of the hydrated (hydrolysis-accelerating) form HFFOH. Under these conditions then it is obvious that the velocity of hydrolysis would be directly proportional to the concentration of the ferment, which is experimentally the case. And similarly the monomolecular formula would hold good if reaction I proceeded at a very great velocity compared with reaction II, which is a logical deduction from experimental and theoretical evidence.

It has been pointed out that the velocity constant gradually decreases as the concentration of the substrate increases. The above hypothesis also offers a reasonable explanation of this fact.⁸⁴ According to the above scheme for the process of hydrolysis the enzyme must cause a greater or smaller shifting in the station of equilibrium between the protein and its products. At high ferment concentrations the enzyme accelerates hydrolysis of the protein more than its synthesis because the hydrolysis-accelerating form of the enzyme is initially present in considerable excess of the synthesis-accelerating form, but at high concentrations of substrate the protein accelerates the dehydration of the enzyme more than its hydration because it is initially present in great excess of its products of hydrolysis. This latter condition will, therefore, cause a slowing of the velocity of hydrolysis which is experimentally found to occur.

The question of synthesis is quite a different problem and one that does not concern us here. The reaction of synthesis being a reaction of the second or a higher order a decided shift in the

⁸³ Euler *Zeitschr f physiol Chem*, lii, p 146, 1907

⁸⁴ See T Brailsford Robertson *Die physikalische Chemie der Proteine* p 408, Dresden, 1912, Verlag von Theodor Steinkopff

station of equilibrium between the Products \rightarrow Protein in the direction of synthesis by the addition of large quantities of enzyme, and also shifting the position of equilibrium between the hydrated and the anhydrous form of the enzyme in the direction of the latter by the presence of large amounts of the products of hydrolysis or by increasing the temperature, must be brought about before the reaction will proceed in the direction of synthesis

The foregoing experiments may be briefly summarized as follows

1 The method of estimating the velocity with which a protein (casein) is hydrolyzed by determining the nitrogen in the undigested portion after precipitation with acetic acid yields results admitting of an accurate physico-chemical interpretation

2 Upon the addition of a slight excess of alkali to neutral or faintly alkaline solutions of casein immediately before precipitation with acetic acid, precipitation is hastened and a clear filtrate is assured

3 The relation between the time of hydrolysis and the amount of "basic" sodium caseinate hydrolyzed, is, for all stages of the reaction, what would be expected from the monomolecular formula $\text{Log}_{10} \frac{a}{a-x} = Kt$

4 The velocity with which "basic" sodium caseinate is hydrolyzed by trypsin is directly proportional to the concentration of the ferment

5 There is a general proportionality between the concentration of the substrate and the velocity of hydrolysis, although the velocity constant decreases slightly as the concentration of the substrate increases

6 The nature of the base combined with casein has little or no influence in the process of hydrolysis "Basic" caseinates of Li, Na, K, NH_3 , Ca, Sr, and Ba hydrolyze with approximately equal velocities for all concentrations of substrate between 0.4 and 2 per cent

7 There is no relation between the degree of dissociation and the rate with which "basic" caseinates are hydrolyzed by trypsin, as in the solutions employed the caseinates of the alkalis are almost completely dissociated while the caseinates of the alka-

line earths are only about 50 per cent dissociated, yet both series of "salts" are hydrolyzed by trypsin at approximately the same velocity

8 There is evidence of rapid autohydrolysis in solutions of "neutral" and "basic" caseinates of the alkalies and alkaline earths

Finally, I wish to express my appreciation of the valuable advice and incessant interest of Dr T Brailsford Robertson at whose suggestion this work was undertaken and with whose aid it has been accomplished

ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS VII SALMINE

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A THE PREPARATION OF SALMINE SULPHATE

A member of the protamine group, namely salmine¹ was prepared by the method of Kossel² as follows

The ripe testicles of the Pacific salmon were minced and the macerated mass which was thus obtained was shaken up in tall glass cylinders with five or six times its volume of distilled water. The thick suspension of sperm which was thus obtained was syphoned off from the supernatant connective tissue and curdled by the addition of 80 cc per liter of $\frac{N}{10}$ acetic acid. The curdled mass of sperm was then washed in ten times its volume of 95 per cent alcohol and this washing was repeated twice, it was then washed once in the same volume of absolute alcohol and then in the same volume of ether. The powder, wet with ether, which was thus obtained, was spread out upon filter paper to dry in the air in a warm dry place.

Each 15 grams of the dried sperm was then stirred up in 350 cc of 1 per cent by volume H_2SO_4 , for about six hours. This mixture was then filtered through hardened filter paper and the filtrate obtained from the extraction of 15 grams of sperm was placed in a tall glass cylinder of about 4000 cc capacity which was then filled with absolute alcohol. After allowing the precipitate to settle, the supernatant fluid was syphoned off, the precipitate contained in two cylinders was collected in one and this was filled with alcohol again.³ The entire precipitate, suspended in alcohol, from the extract

¹ According to Taylor (*Univ of Calif Publ Pathol*, 1, p 7, 1904) the protamine which is contained in the sperm of the Pacific salmon is identical with the salmine found in the sperm of the European salmon.

² A Kossel *Zeitschr f physiol Chem*, xxv, p 165, 1898

³ It is necessary to avoid washing with alcohol too frequently, as on suspending the protamine sulphate in alcohol for a third or fourth time a very stable suspension is produced from which protamine is only deposited very slowly.

of 300 grams of sperm, was dissolved by the addition of about 4 liters of hot water (about 80°C), the least soluble portion was filtered off, and the remainder reprecipitated by the addition of 10 volumes of alcohol. This precipitate was washed once in the same volume of alcohol as that employed in precipitation and then in a like volume of ether. The final suspension of protamine sulphate in ether, obtained after syphoning off the supernatant ether, was collected in a hardened filter, dried over sulphuric acid at 40° for two days and then pulverized and sifted. The product is a friable white powder. The yield from 300 grams of sperm was 14.6 grams.

The empirical formula of the substance which is thus obtained is according to Kossel (*loc cit*) and Taylor (*loc cit*), $\text{C}_{30}\text{H}_{51}\text{N}_7\text{O}_4 \cdot 2\text{H}_2\text{SO}_4$. It readily dissolves in water up to about 2 per cent at 20° . It diffuses through parchment paper (Taylor). Its solutions are very faintly acid in reaction. According to Taylor the acidity of a 0.5 per cent solution, measured by the gas-chain, is $\frac{N}{300}\text{H}^+$, I found that one gram of my preparation in 0.25 per cent solution required the addition of 9.6 cc of $\frac{N}{10}\text{KOH}$ to render the solution just alkaline to rosolic acid, corresponding, in 0.5 per cent solution to an acidity of less than $\frac{N}{200}$, since an acidity determined by titration in protein solutions must obviously be considerably in excess of the true acidity, it may be inferred that the acidities of solutions of my preparation were not appreciably in excess of Taylor's estimate cited above. Since a 1 per cent solution of protamine sulphate contains 0.0424 equivalents of H_2SO_4 per liter, it is evident that protamine sulphate does not, in aqueous solution, undergo hydrolytic dissociation to any very appreciable extent. According to Taylor, a perfectly neutral preparation of protamine sulphate may be obtained by a special and lengthy process of preparation and purification.

B THE PREPARATION OF SALMINE CHLORIDE

Several attempts were made to prepare salmine carbonate according to the method recommended by Taylor (*loc cit*), in order to prepare the chloride from this substance. Many difficulties were found to attend this procedure, however. If excess of $\text{Ba}(\text{OH})_2$ be added to a dilute solution of protamine sulphate great difficulty is encountered in removing this excess by means of CO_2 even at 50°C . After several hours' passage of CO_2 clear filtrates can be obtained which contain barium, a fact which is probably attribu-

table to the formation of the barium salt of a carbamino derivative of the protamine⁴

Moreover, as Taylor points out, great difficulty is experienced in obtaining clear filtrates, indeed I have found the only successful method to consist in filtration under pressure through a Chamberland filter, a process which is attended by considerable loss of the protamine, since it is, to some extent, retained by the filter⁵

Accordingly, salmine chloride was prepared directly from the sulphate in the following manner

To a carefully weighed amount (1.48 grams) of protamine sulphate dissolved in 100 cc of water was added an exactly sufficient weight of carefully chosen barium chloride crystals, dissolved in about 20 cc of water, to precipitate the H_2SO_4 in the protamine sulphate. This mixture was then set aside in a tall glass cylinder at 50° for twenty-four hours at the end of which time a compact precipitate of barium sulphate had settled to the bottom of the cylinder from which the clear supernatant fluid could readily be decanted. This fluid was filtered through a hardened filter and the protamine chloride precipitated by the addition of 5 to 6 volumes of absolute alcohol. After allowing the precipitate to settle the supernatant fluid was syphoned off and the precipitate was washed in 1 liter of absolute alcohol and twice in 1 liter of ether (über Natrium destilliert) and was finally collected on a hardened filter and dried over H_2SO_4 at 36° for twenty-four hours. It was then pulverized and sifted and dried for another twenty-four hours. The yield was only about a third of a gram, which is attributable to the fact that the precipitate, after washing in alcohol only settled very incompletely, a phenomenon which appears to be characteristic of very anhydrous (or, as Taylor believes, very highly purified) preparations of salmine.

The empirical formula of this substance is, according to Kossel $\text{C}_{30}\text{H}_{51}\text{N}_{17}\text{O}_6 \cdot 4\text{HCl}$. It dissolves readily in water, yielding very faintly acid solutions.

C THE DETERMINATIONS OF REFRACTIVITY

Portions of a 2 per cent solution of salmine sulphate were diluted to 1.5 per cent and to 1 per cent and the refractive indices of these solutions and of water were measured at 22°C in a Pulfrich refractometer, using a sodium flame as the source of light. On another

⁴ M. Siegfried, *Ergeb. d. Physiol.*, ix, p. 334, 1910.

⁵ This is true also of protamine chloride. A 1 per cent solution of protamine chloride, after filtration through a porcelain filter under pressure, was found to be reduced in concentration to about 0.5 per cent.

310 Refractive Indices of Salmine Solutions

occasion a 1 per cent solution was diluted to 0.5 per cent and the refractive indices of these solutions were measured at the same temperature

The following were the results obtained. The values headed a are calculated from the formula $n - n_1 = a \times c$ where n is the refractive index of the solution, n_1 that of the solvent, and c is the percentage of salmine sulphate or of salmine in the solution

TABLE 1

SOLUTION	n =REFRACTIVE INDEX OF SOLUTION AT 22° C	a FOR SALMINE SULPHATE	a FOR SALMINE
Distilled water	1.33410		
H ₂ SO ₄ = 1 per cent salmine sulphate = 42.4 cc $\frac{N}{10}$ H ₂ SO ₄ per 100 cc	1.33450		
0.5 per cent salmine sulphate	1.33497	0.00174 ± 0.00007	0.00173 ± 0.00009
1.0 per cent salmine sulphate	(1) 1.33584		
1.0 per cent salmine sulphate	(2) 1.33584		
1.5 per cent salmine sulphate	1.33671		
2.0 per cent salmine sulphate	1.33759		

The calculation of a for salmine sulphate is performed by adding together all observed values of $n - n_1$ and dividing this sum by the sum of the percentages of salmine sulphate employed. That for salmine is calculated upon the assumption that 1 gram of salmine sulphate contains 0.792 grams of salmine and that when the refractivity of the sulphuric acid in the compound be subtracted from the observed values of $n - n_1$, the remainders represent the refractivity of salmine. The details of these calculations follow

TABLE 2

PERCENTAGE OF SALMINE SULPHATE	PERCENTAGE OF SALMINE	$n-n_1$ FOR SAL- MINE SULPHATE	$n-n_1$ FOR SALMINE	POSSIBLE ERROR IN DETERMINATION OF $n-n_1$
2 0	1 484	0 00349	0 00269	0 00008
1 5	1 188	0 00261	0 00201	0 00008
1 0	0 792	0 00174	0 00134	0 00008
1 0	0 792	0 00174	0 00134	0 00008
0 5	0 396	0 00087	0 00067	0 00008
Sums 6	4 652	0 01045	0 00805	≈ 0 00040

It will be observed that the numbers enumerated in the third column of the above table, divided by those in the first column, yield a constant quotient. We may therefore conclude that *the change in the refractive index of water which is brought about by dissolving salmine sulphate therein is directly proportional to the concentration of the salmine sulphate*.

A 0.5 per cent solution of salmine chloride was prepared and its refractive index and that of water were determined at 18° C. The following was the result obtained.

SOLUTION	n = REFRACTIVE INDEX OF SOLUTION AT 18° C	α FOR SALMINE CHLORIDE
Distilled water	1 33356	
One-half per cent chlo- ride	1 33442	0.00172 ± 0.00016

A 1 per cent solution of salmine chloride contains 0.0446 N hydrochloric acid and 0.837 grams of salmine. Hence in a 1 per cent solution of salmine chloride the refractivity of the hydrochloric acid (calculated by interpolation from the refractivity of a $\frac{N}{16}$ solution of HCl) is 0.00032.

From this and the above value of α for salmine chloride it follows that the change in the refractive index of water due to the introduction of 0.837 per cent of salmine in the form of its chloride is

$$0.00172 \pm 0.00016 - 0.00032 = 0.00140 \pm 0.00016$$

Hence the change in the refractive index of water due to the introduction of *one per cent* of salmine in the form of its chloride is

$$0.00167 \pm 0.00019$$

Thus, within the experimental error, *the refractivity of salmine in solutions of salmine chloride is identical with its refractivity in solutions of salmine sulphate*

CONCLUSIONS

1 The change in the refractive index of water which is brought about by dissolving salmine sulphate therein is directly proportional to the concentration of the dissolved salmine sulphate

2 The value of a (= change in the refractive index of the solvent due to the introduction of 1 per cent of the protein) for salmine sulphate is 0.00174 ± 0.00007

3 The value of a for salmine chloride is 0.00172 ± 0.00016

4 The value of a for the base salmine when combined to form salmine sulphate is 0.00172 ± 0.00009

5 The value of a for salmine in the form of salmine chloride is, within the above experimental error, identical with its value for salmine in the form of salmine sulphate

STUDIES ON THE EFFECT OF LECITHIN UPON THE FERMENTATION OF SUGAR BY BACTERIA

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(Received for publication, March 5, 1912.)

This work was undertaken with a view to studying the effect of lecithin upon the process of sugar fermentation *in vitro*. Many functions have been ascribed to lecithin, and considerable discussion has arisen of late concerning its rôle in metabolism. It has been assumed by a number of investigators, that lecithin exerts an inhibitory action upon the oxidative processes in the animal body.

Diabetes was at one time believed to be due to the inhibitory action of lecithin on the oxidation of sugar. This hypothesis has been chiefly upheld by Lùthje, who found that the sugar output in diabetics was usually increased by the administration of egg yolk, a substance rich in lecithin. Bang, as is known, suggested that lecithin and dextrose combined in the blood, forming a substance called jecorin.

Some have even asserted that the alleged decrease in the intrinsic or fundamental metabolism, and the decrease in the oxygen consumption occurring in adiposis, are attributable to the presence of a larger amount of lecithin in the body fluids than that normally present. For example, Kempner and Schepilewsky¹ found that white mice usually increased in weight after receiving injections of lecithin.

Acting upon this belief, Russian and French investigators have suggested the use of lecithin therapeutically in cases of emaciation and wasting diseases. In support of their claims, the authors quote a number of experiments made upon animals, the results of which do not appear as convincing as the authors believe them to be.

¹ Kempner and Schepilewsky. *Zeitschr. f. Hyg.*, LVII, p. 213, 1898.

Lately Yoshimoto² made a number of animal experiments with lecithin, and instead of finding a diminished output of nitrogen in the urine, he found it to be increased—the increase being the exact equivalent of the nitrogen present in the lecithin administered to the animals. This investigator, therefore, came to the conclusion that lecithin does not exert an inhibitory action on metabolism.

The attempt to ascertain any such function of lecithin *in vivo* is naturally associated with many difficulties. Even were we to find a diminution in the nitrogen output after feeding lecithin to animals, the conclusion that lecithin produced this result by virtue of its action upon nitrogenous metabolism, would not be justifiable. Such a result might be due to an indirect action, for example, we could readily conceive of an increased mobilization of fats produced by lecithin, or possibly an increased oxidation of sugars, the result of which would be a sparing of protein material, leading consequently to a diminished output of nitrogen in the urine. This effect would manifestly be, not the result of inhibitory action of lecithin upon nitrogenous metabolism, but the indirect result of an increased combustion of fats and sugars.

The difficulties which arise in the study of a problem of this character *in vivo*, are chiefly due to the fact that it is almost impossible to dissociate a single function or chemical process from every other in the body.

It seemed, therefore, desirable to approach the subject in a somewhat simpler way. Vallet and Rimbaud,³ as well as Renshaw and Atkins,⁴ have recently attempted to solve the problem of the rôle of lecithin in biological processes by studying its effect upon the growth of bacteria. Their results show that lecithin does not materially influence the growth of bacteria. However, the method pursued by the above investigators, when taken in conjunction with the object sought after, is not free from criticism. Cellular growth and cellular function should not be confused, the one need not be an index of the activity of the other. The protoplasm of many cell and tissue-forms is endowed with some of the functions present in the living cells. This is true of

² Yoshimoto *Zeitschr f physiol Chem*, lxiiv, p 464, 1910

³ Vallet et Rimbaud *Compt rend soc biol*, lxxviii, p 302, 1910

⁴ Renshaw and Atkins *Journ Amer Chem Soc*, pp xcvi-xcviii, 1910

many enzyme or ferment-bearing cells The extract of the yeast cell, for example, can ferment sugar as does the living cell itself

Here we have a function that is resident in the cell material and is active even in the absence of cell life A number of experiments have been made by Kuettner,⁵ and also by O Schwarz,⁶ with lecithin and the different digestive ferments Schwartz, for example, ascribes the inhibitory action of blood serum upon trypsin, to lipoids, presumably lecithin

We have therefore, deemed it necessary to take up the study of lecithin in connection with the fermentation of sugars by bacteria In so doing we deal with a single comparatively simple process The character of the agents used in the tests to produce the fermentation, is also relatively simple

For our purposes we used three types of bacteria, namely, *Bacillus coli communis*, *Bacillus mucosus capsulatus*, and *Bacillus acidilactici* Each of these organisms ferments certain sugars Our measure of bacterial activity, therefore, was the production of gas and acid

The amount of lecithin employed in our media at no time exceeded 0.4 per cent This, of course, is an amount of the substance in excess of that found in biological fluids If, therefore, lecithin could modify oxidative processes in respect to sugar fermentation, then it would manifest itself by an increase or decrease in the production of gas or acid, or both We tested the fermentative action of the above bacteria on twelve different types of sugars This was done to ascertain whether or not the chemical constitution of sugar played any rôle in the rate and character of its decomposition by bacteria

The list of sugars includes the alcohol, aldehyde, and ketone type of the different saccharides (mono-, di-, and polysaccharide) A hexavalent alcohol and an aldehyde pentose are also represented in the series

A 1 per cent solution of each sugar in nutrient bouillon (neutral to phenolphthalein) was used The media were distributed into fermentation and straight test tubes, 10cc of each medium being used as the unit To one set of tubes, a 4 per cent emulsion of

⁵ Kuettner *Zeitschr f physiol Chem*, 1, p 472, 1906-07

⁶ Schwarz *Wien Klin Wochenschr*, 1909

lecithin was added, allowing 1 cc for each tube To the other set, sterile salt solution was added in like amount

Both sets of tubes were inoculated with a loopful of an emulsion of each bacterium and incubated at 37.5°C All the tests were made duplicate A parallel series of uninoculated tubes were incubated and used as controls

The amount of gas produced in the fermentation tubes was recorded in cubic centimeters at the end of twenty-four and forty-eight hours incubation The following table shows the results obtained in tests on gas production

TABLE I
Showing gas production in cubic centimeters

	MONOSACCHARIDES			DIBACCHARIDES			TRI-SACCHARIDE	POLY-SACCHARIDES		TRIVALENT ALCOHOL	HEXA-VALENT ALCOHOL	ALDEHYDE PENTOSE
	Dextrose	Galactose	Levulose	Maltose	Lactose	Saccharose		Inulin	Dextrin			
<i>Bacillus coli communis</i>												
Plain medium { 24 hours	10	10	10	20	20	05	20				30	10
Plain medium { 48 hours	10	20	20	30	20	10	20				40	10
Plain medium { 24 hours	10	20	10	20	30	10	05				30	10
Plain medium { 48 hours with lecithin	20	30	20	30	40	20	05				10	10
<i>Bacillus mucosus capsulatus</i>												
Plain medium { 24 hours	20	20	20	20	20						30	20
Plain medium { 48 hours	25	20	20	25	20						30	30
Plain medium { 24 hours	10	20	20	20	10						30	10
Plain medium { 48 hours with lecithin	20	30	30	25	10						10	20
<i>Bacillus acidilactici</i>												
Plain medium { 24 hours	20	30	40	20	20	20	30				30	10
Plain medium { 48 hours	20	50	40	20	50	40	35				40	25
Plain medium { 24 hours	10	20	20	20	20	40	10				10	15
Plain medium { 48 hours with lecithin	30	30	30	30	40	50	25				40	35

SUMMARY

Bacillus coli communis

INCREASED GAS	DECREASED GAS	NO EFFECT
Dextrose	Raffinose	Levulose
Galactose		Maltose
Lactose		Inulin
Saccharose		Dextrin
		Glycerine
		Mannit
		Arabinose

Bacillus mucosus capsulatus

Galactose	Dextrose	Maltose
Levulose	Lactose	Saccharose
Mannit	Arabinose	Raffinose
		Inulin
		Dextrin
		Glycerine

Bacillus acid lactici

Maltose	Galactose	Dextrose
Saccharose	Lactose	Inulin
Dextrin	Levulose	Glycerine
Arabinose	Raffinose	

In the above table we note that with *Bacillus coli communis* lecithin favors an increase in gas formation in the monosaccharides dextrose and galactose, and the disaccharides lactose and saccharose, while it inhibits gas formation in the trisaccharide raffinose, the remaining sugars are unaffected

Lecithin aids gas production by *Bacillus mucosus capsulatus* in the monosaccharides galactose and levulose, while it arrests gas production with dextrose Lactose and arabinose are influenced in like manner

With *Bacillus acid lactici* lecithin also aids the formation of gas in the disaccharides maltose and saccharose, while it checks gas fermentation in all the monosaccharides, excepting dextrose, in the dissacharide lactose, and in raffinose and in mannit The remaining media are unaffected

TABLE 2 (a)
Showing acid production in the different sugar media
 In cubic centimeters of $\frac{N}{10}$ NaOH

	MONOSACCHARIDES			DISACCHARIDES			TRI-SACCHARIDE	POLY-SACCHARIDE		TRIVALENT ALCOHOL	HEXA-VALENT ALCOHOL	ALDHYDE PENTOSE
	Dextrose	Galactose	Levulose	Maltose	Lactose	Saccharose		Inulin	Dextrin			
Plain medium-control	0 2	0 35	0 7	0 35	0 0				0 15			0 05
Plain medium B coli minus	7 1	4 0	5 1	1 4	5 2			0 4	0 12		4 2	3 00
Net acid produced	6 9	4 25	4 4	1 05	4 0		1 05	0 4	0 03		4 2	2 95
Plain med B muc caps	0 15	0 35	4 3	0 5	0 0				0 02			0 45
Net acid produced	0 25	0	3 0	0 15	0 3				0 05			0 20
Plain med B acid lact	4 0	5 5	1 8	7 6	4 0	1 8		2 85	4 5		6 15	7 85
Net acid produced	3 8	5 15	4 1	7 25	4 3	1 8	1 7	2 85	4 35		0 15	7 20

TABLE 2 (b)
Showing acid production on the sugar media in the presence of 0.4 per cent lecithin

	MONOSACCHARIDES			DISACCHARIDES			TRI-SACCHARIDE	POLY-SACCHARIDE		TRIVALENT ALCOHOL	HEXA-VALENT ALCOHOL	ALDEHYDE PENTOSE
	Dextrose	Galactose	Levulose	Maltose	Lactose	Saccharose	Raffinose	Inulin	Dextrin	Glycerine	Mannit	Arabinose
Lecithin medium control	1.75	1.6	1.45	1.4	1.9	0.3		0.5	1.35	0.3	0.95	2.0
Culture <i>B. coli</i> communis	4.5	5.8	7.85		6.3	1.2		1.85	1.5	0.1	7.55	5.1
Net acid produced	2.75	4.2	5.4		4.4	0.9		1.35	0.15	-0.2	6.65	3.1
Cult. <i>B. muc. caps</i>	6.1	2.15	8.35		2.6	0.5		0.35	0.95		0.9	4.1
Net acid produced	4.35	5.5	6.9		0.7	-0.2		-0.15	-0.4	-0.3	-0.05	3.9
Cult. <i>B. acid lact.</i>	9.1	9.4	7.8	12.5	10.5	12.0	9.8	5.3	6.70		7.4	9.5
Net acid produced	7.35	7.80	6.35	11.1	8.7	11.7	9.8	4.8	5.35		6.45	7.5

As the above tables indicate, the results of acid production are more uniform than those obtained with gas production. *Bacillus coli communis* causes an increased acid production in the presence of lecithin with all the sugars, excepting dextrose and raffinose. With the latter two sugars this bacillus produces less acid in the presence of lecithin than otherwise.

Bacillus mucosus capsulatus produces more acid in the presence of lecithin than otherwise, with all the sugars excepting glycerine and raffinose, upon the latter lecithin has no effect. *Bacillus acidilactici* in the presence of lecithin produces more acid in all the sugar media.

It is significant that in all the tests for acidity, lecithin has a distinct tendency to increase rather than to decrease the acid production, and if we take acid-production as an index of oxidative processes, then we must conclude that lecithin aids oxidation of the sugars.

Lecithin itself contains certain radicles which on decomposition yield acid and gas. Its one constituent, cholin, can (as Hasebroek⁷ has shown) on anaërobic putrefaction be split into carbon dioxide, methane, ammonia and methylamine. Although the conditions existing in our tests and those which occur in anaërobic putrefaction are totally different, nevertheless the question might be asked, whether or not, a decomposition of lecithin in the presence of the bacteria takes place, which may be responsible, at least in part, for the results recorded. Tests were therefore instituted to answer this particular question. Cultures were made of the organisms in sugar-free bouillon, with and without lecithin, and, on comparing the results obtained, it was found that the bacteria employed do not cause any acid or gas production from lecithin, even after seventy-two hours' incubation.

It is necessary to call attention to the fact that in all our tests, lecithin is presumably present in a free state, and although we have reason to believe from the work done by one of the authors (E) in another connection, that lecithin enters into combination with peptone bodies, such as are present in our culture media, we must for the present infer that the lecithin present is in a free state, and the conclusions to be drawn must apply to the action of lecithin present in this state.

⁷ Hasebroeck. *Zeitschr f physiol Chem*, xii, p 148, 1888

To summarize briefly, our conclusions, therefore, are (1) Free lecithin may modify the bacterial fermentation of different sugars, hence, oxidative processes (2) The action of lecithin increases the fermentation of some sugars and lessens that of others There is apparently no definite relationship between the action of lecithin upon sugars and their chemical composition

To sum up the tendency of lecithin is to increase rather than to decrease fermentation

THE BALANCE OF ACID-FORMING AND BASE-FORMING ELEMENTS IN FOODS, AND ITS RELATION TO AMMONIA METABOLISM

BY H C SHERMAN AND A O GETTLER

(Contribution from the Havemeyer Laboratories of Columbia University,
No 205)

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In recent years the ash constituents of foods have come to hold an increasingly prominent place in considerations of food values. It is now generally recognized not only that the food as a whole should supply adequate amounts of each of the chemical elements which is essential to the body structure, but also that these elements should stand in normal quantitative relations to each other.

Conspicuous among the quantitative relations is that between the acid-forming and the base-forming elements of the food. It has long been known that certain foods contain a surplus of base-forming over acid-forming elements as evidenced by the fact that on burning they yield a strongly alkaline ash, whereas other foods lose acid-forming elements in ashing and yet yield a neutral ash showing that acid-forming elements must have predominated in the food.

It was, however, not possible to make any useful quantitative comparisons on the basis of the data which had been obtained by the usual methods and recorded in the accepted tables of ash analyses, because these data represented only the composition of the material which remained after ignition, regardless of the fact that in many cases a large part of the acid-forming elements exist in the food as constituents of the organic matter and pass off during the ignition. This is particularly true of sulphur, which, so far as known, exists in foods chiefly as a constituent of protein and often is expelled almost entirely during the burning,

so that the sulphates found in the analysis of the ash represent only a very minor part of the sulphur which was present in the food. Hence any attempt to calculate the relation of acid-forming to base-forming elements from the tables of ash analyses which have been available in the past would give erroneous results except in those cases in which the base-forming elements so far predominate as to prevent loss of acid-forming elements during ashing, and comparisons of different types of food with each other would be misleading.

Another source of error lies in the fact that the usually accepted tables of ash analyses are largely derived from the work of agricultural chemists whose primary object was to determine the constituents removed from the soil by the crop and who, therefore, analyzed the article of food as sold instead of simply the edible portion.

An investigation of the actual quantitative relations of acid-forming and base-forming elements in the edible matter of foods was begun in this laboratory over five years ago and a few of the results first obtained were published in the summer of 1907.¹

We are now able to present data for a larger number of foods. Of some of these we have made complete ash analyses, in other cases we have accepted previously published results for such constituents as seemed to have been accurately determined and supplemented these with such determinations as were necessary. While it is obviously more systematic to balance the acids and bases by comparisons of data obtained upon the same sample, the compiled data sometimes have the advantage of representing the average of several samples. Hence in Table 1 we give in some cases the results calculated from data partly compiled even though we may have determined the complete data upon a single specimen of the same food. Footnote references attached to the names of the articles of food show the nature of the data in each case. In most of our own work the material selected for analysis consisted of composite samples each representing a mixture of several specimens of the particular food.

METHODS OF ANALYSIS —For the determination of *calcium* and *magnesium* the sample was burned in a platinum dish (usually in a muffle), the ash

¹ Sherman and Sinclair. *This Journal*, III, p. 307.

dissolved in hot, very dilute hydrochloric acid, the solution filtered if not clear, treated with oxalic acid, heated to boiling, a few drops of methyl orange added as indicator, and then ammonia very gradually until the indicator changed color. This neutralization usually required about one-half hour. Finally an excess of ammonium oxalate was added and the solution allowed to stand for four hours, then filtered, washed with one per cent solution of ammonium oxalate, dried, ignited to constant weight and weighed as calcium oxide, with care to avoid absorption of carbon dioxide or moisture.

The filtrate from the calcium oxalate was evaporated to dryness in platinum and ignited carefully to expel ammonium salts without spattering, the residue dissolved in hot water with the addition of a little hydrochloric acid, the solution filtered if necessary, cooled, and the magnesium precipitated by adding acid sodium phosphate solution and then ammonia gradually until distinctly alkaline. Half an hour later an excess of ammonia was added as usual and after allowing to stand in the cold usually for about 12 hours the ammonium magnesium phosphate was filtered, washed with dilute ammonia, ignited to magnesium pyrophosphate and weighed.

For the determination of sodium and potassium a portion of the sample was burned in platinum at a low temperature (very dull redness) to a white ash. This was taken up with hot water and a little hydrochloric acid, filtered, heated to 95° and treated with a very slight excess of barium chloride solution (added drop by drop with constant stirring), allowed to stand on water bath for one hour, then without filtering, barium hydroxide solution was added in the same manner to alkaline reaction, the solution filtered and residue washed free from chlorides. The filtrate was treated with a few drops of ammonia and an excess of ammonium carbonate, filtered, the filtrate and washings evaporated to dryness, ignited carefully at very dull redness, cooled, dissolved in hot water containing a few drops of hydrochloric acid, filtered, and again evaporated and ignited as before. This purified residue was weighed as sodium and potassium chlorides, then dissolved and the potassium determined by the usual platonic chloride method.

In the determination of phosphorus the organic matter was usually oxidized by boiling with nitric and sulphuric acids in a Kjeldahl flask. In order to avoid error from incomplete destruction of phosphatids or from subsequent incomplete precipitation of ammonium phospho-molybdate it is well to use only 10 cc of concentrated sulphuric acid, then after the organic matter appears to have been largely destroyed add 10 grams of ammonium nitrate and heat until the solution in the flask is reduced to 10 cc or less, thus ensuring a high temperature which should char any phosphatids which may not have been destroyed by the boiling mixture of sulphuric and nitric acids, if charring occurs more nitric acid is added and the boiling repeated. Finally the acid solution was washed out of the flask, treated with 20 grams ammonium nitrate and the phosphoric acid determined by precipitation first as ammonium phospho-molybdate and subsequently as magnesium ammonium phosphate according to the well-known gravimetric method. In some cases phosphorus was also determined after burning the sample with sodium carbonate and potassium nitrate, this giving the same results as the

method of decomposition with acids when both methods are properly performed

For the determination of *chlorine* the sample was burned at a low temperature with a liberal excess of sodium carbonate in a room kept as free as possible from hydrochloric acid or ammonium chloride fumes, and the chloride was determined gravimetrically by precipitation as silver chloride

The oxidation of organic matter for the determination of *sulphur* was accomplished sometimes by burning in oxygen in a bomb calorimeter as first suggested by Berthelot, but usually by heating the substance in a nickel crucible with sodium hydroxide and sodium peroxide essentially according to the methods of Osborne and of Folin with such variations in manipulation as were found best adapted to the behavior of the different types of food. Earlier experiments by one of us,² had shown that the compressed oxygen method and the peroxide method are both capable of yielding accurate results when properly carried out. After the complete destruction of organic matter and oxidation of sulphur to sulphates, the determination of the latter was made by precipitation as barium sulphate in the usual manner with careful attention to the established precautions

Table 1 shows the percentages of calcium, magnesium, sodium, potassium, phosphorus, chlorine, and sulphur in the edible portion of a considerable number and variety of food materials

In order to balance the acid-forming against the base-forming elements we have calculated the volume of a normal acid or alkali solution which would correspond to the amount of each element in 100 grams of the food material, phosphoric acid being calculated as a dibasic acid. By adding together the results obtained for all of the base-forming and for all of the acid-forming elements respectively and comparing the totals we find the excess of acid-forming or of base-forming elements in terms of cubic centimeters of a normal solution per 100 grams of edible food material

Since, however, the different food materials vary so greatly in their moisture content and food value it may give a more serviceable impression of the relative acid-forming or base-forming tendencies of different food materials if the surplus acid or base be stated for 100 calorie portions rather than for 100 gram portions of the various foods

Table 2 calculated from the data given in Table 1 shows the excess of acid-forming over base-forming elements or *vice versa* both per 100 grams and per 100 calories of edible food material

It will be seen from the above tables that all the meats (in-

² Sherman *Journ Amer Chem Soc*, XLIV, p 1100

TABLE I

Percentages of Calcium, Magnesium, Potassium, Sodium, Phosphorus, Chlorine and Sulphur in edible portion of Foods

ARTICLE OF FOOD	CAL- CIUM	MAG- NESIUM	SODIUM	POTAS- SIUM	PHOS- PHORUS	CHLOR- INE	SUL- PHUR
	percent	percent	percent	percent	percent	percent	percent
Almonds*	0 270	0 275	0 024	0 756	0 496	0 037	0 185
Almonds†	0 215	0 211	0 022	0 166	0 379	0 005	0 135
Apples†	0 010	0 008	0 015	0 125	0 013	0 004	0 005
Asparagus†	0 029	0 012	0 007	0 165	0 039	0 040	0 040
Bananas†	0 007	0 024	0 015	0 415	0 024	0 200	0 013
Beans, dried*	0 165	0 167	0 189	1 428	0 453	0 007	0 214
Beans, dried†	0 157	0 151	0 193	1 162	0 497	0 030	0 220
Beans, lima, dried†	0 071	0 187	0 245	1 743	0 336	0 025	0 160
Beans, lima, fresh†	0 029	0 066	0 089	0 581	0 118	0 009	0 060
Beets†	0 021	0 020	0 074	0 374	0 039	0 040	0 015
Cabbage*	0 049	0 014	0 020	0 243	0 027	0 013	0 067
Cabbage†	0 049	0 016	0 037	0 374	0 039	0 030	0 070
Carrots†	0 055	0 021	0 096	0 291	0 044	0 036	0 022
Cauliflower†	0 122	0 012	0 074	0 224	0 061	0 050	0 085
Celery†	0 071	0 024	0 082	0 307	0 044	0 170	0 025
Cherry juice†	0 018	0 012	0 015	0 125	0 013	0 004	0 006
Chestnuts†	0 029	0 048	0 037	0 415	0 087	0 010	0 068
Corn, sweet dried, †	0 021	0 121	0 148	0 415	0 349	0 050	0 160
Crackers*	0 050	0 059	0 580	0 117	0 111	0 857	0 193
Currants, dried†	0 036	0 024	0 015	0 208	0 044	0 010	0 010
Eel†	0 039	0 018	0 032	0 241	0 177	0 035	0 135
Eggs†	0 067	0 009	0 148	0 137	0 161	0 100	0 190
Egg white†	0 011	0 009	0 155	0 158	0 013	0 150	0 196
Egg yolk†	0 143	0 012	0 074	0 108	0 044	0 100	0 157
Fish, haddock†	0 022	0 017	0 099	0 335	0 137	0 241	0 223
Fish, pike†	0 040	0 031	0 029	0 416	0 213	0 032	0 218
Lemons†	0 036	0 006	0 007	0 174	0 009	0 010	0 012
Lettuce†	0 036	0 006	0 030	0 348	0 039	0 060	0 014
Meat, beef, lean I*	0 014	0 035	0 035	0 359	0 210	0 061	0 237
Meat, beef, lean II*	0 016	0 024	0 082	0 341	0 193	0 048	0 214
Meat, beef, lean†	0 008	0 024	0 067	0 348	0 218	0 050	0 200
Meat, beef, lean†	0 002	0 024	0 065	0 366	0 170	0 057	0 187
Meat, chicken†	0 011	0 037	0 095	0 465	0 258	0 060	0 292
Meat, frog†	0 016	0 024	0 055	0 308	0 186	0 040	0 163
Meat, pork, lean†	0 008	0 028	0 156	0 254	0 213	0 048	0 204
Meat, rabbit†	0 018	0 029	0 046	0 398	0 253	0 051	0 199

Data determined in this laboratory

† Data partly compiled partly determined in this laboratory

‡ Data published by Katz

TABLE I—Continued

ARTICLE OF FOOD	CAL- CIUM	MAG- NESIUM	SODIUM	POTAS- SIUM	PHOS- PHORUS	CHLOR- INE	SUL- PHUR
	percent	percent	percent	percent	percent	percent	percent
Meat, veal†	0 014	0 030	0 086	0 380	0 220	0 067	0 226
Meat, venison†	0 010	0 029	0 070	0 336	0 249	0 041	0 211
Milk, cow's*	0 124	0 011	0 069	0 154	0 092	0 091	0 031
Milk, cow's†	0 120	0 011	0 051	0 142	0 094	0 120	0 033
Muskmelon†	0 017	0 012	0 061	0 235	0 015	0 041	0 014
Oatmeal*	0 060	0 143	0 072	0 365	0 402	0 027	0 215
Oatmeal†	0 093	0 127	0 081	0 380	0 380	0 035	0 215
Oranges†	0 043	0 012	0 007	0 183	0 022	0 010	0 013
Peaches†	0 007	0 012	0 015	0 208	0 020	0 010	0 010
Peanuts†	0 071	0 169	0 052	0 706	0 392	0 040	0 243
Peas, dried*	0 139	0 150	0 072	0 940	0 370	0 034	0 264
Peas, dried†	0 100	0 145	0 118	0 880	0 397	0 040	0 230
Potatoes I*	0 006	0 022	0 048	0 403	0 042	0 059	0 042
Potatoes II*	0 009	0 027	0 043	0 431	0 047	0 094	0 043
Potatoes†	0 011	0 022	0 019	0 440	0 061	0 030	0 030
Prunes*	0 038	0 046	0 101	0 845	0 080	0 004	0 032
Prunes†	0 043	0 048	0 074	0 996	0 109	0 010	0 030
Radishes†	0 036	0 012	0 082	0 141	0 039	0 050	0 030
Raisins†	0 057	0 009	0 141	0 830	0 126	0 070	0 060
Raspberry juice†	0 021	0 018	0 007	0 141	0 013	0 010	0 007
Rice I*	0 018	0 058	0 109	0 104	0 110	0 133	0 170
Rice II*	0 022	0 044	0 025	0 070	0 080	0 075	0 118
Rice†	0 008	0 027	0 021	0 068	0 089	0 050	0 105
Turnips*	0 023	0 009	0 067	0 101	0 021	0 018	0 046
Turnips†	0 064	0 169	0 059	0 332	0 051	0 040	0 070
Wheat, entire*	0 044	0 170	0 106	0 515	0 469	0 088	0 174
Wheat, entire†	0 044	0 128	0 051	0 431	0 393	0 080	0 170
Wheat, flour*	0 026	0 030	0 069	0 146	0 086	0 076	0 206

* Data determined in this laboratory

† Data partly compiled, partly determined in this laboratory

‡ Data published by Katz

cluding fish) examined show decided excess of acid-forming elements. The meats of different species or of young and mature animals of the same species show very similar results in this respect. The acid-forming elements also predominate in eggs though to a somewhat less degree than in lean meats. Grain products show a much smaller predominance of the acid-forming elements than do meats and eggs when compared on the 100

TABLE 2

Excess of acid-forming or base-forming elements, calculated from Table 1

ARTICLE OF FOOD	EXCESS ACID OR BASE IN TERMS OF NORMAL SOLUTIONS			
	Per 100 grams		Per 100 calories	
	Acid	Base	Acid	Base
	cc	cc.	cc	cc
Almonds*		12 38		1 86
Almonds†		11 76		1 76
Apples†		3 76		5 98
Asparagus†		0 81		3 65
Bananas†		5 56		5 62
Beans, dried*		23 87		6 92
Beans, dried†		11 58		3 36
Beans, lima, dried†		41 65		12 08
Beets†		10 86		23 57
Cabbage*		4 34		13 76
Cabbage†		7 10		22 51
Carrots†		10 82		23 91
Cauliflower†		5 33		17 48
Celery†		7 78		42 17
Cherry juice†		4 40		
Chestnuts†		7 42		3 19
Corn, sweet, dried†	5 95		1 77	
Crackers*	7 81		1 95	
Currants, dried†		5 97		1 85
Eel†	9 89		**	
Eggs†	11 10		7 55	
Egg white†	5 24		9 52	
Egg yolk†	26 69		7 08	
Fish haddock†	16 07		**	
Fish, pike†	11 81		**	
Lemons†		5 45		12 32
Lettuce†		7 37		38 69
Meat, beef, lean, I*	13 91		12 10	
Meat, beef, lean, II*	10 05		8 74	
Meat, beef, lean, †	12 00		10 44	
Meat, beef, lean, †	13 67		11 89	
Meat, chicken†	17 01		**	
Meat, frog†	10 36		**	
Meat, pork, lean†	11 87		**	

Data determined in this laboratory

† Data partly compiled partly determined in this laboratory

‡ Data published by Katz

Data insufficient to permit calculation of acid to calorie basis

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TABLE II—Continued

ARTICLE OF FOOD	EXCESS ACID OR BASE IN TERMS OF NORMAL SOLUTIONS			
	Per 100 grams		Per 100 calories	
	Acid	Base	Acid	Base
	cc	cc	cc	cc
Meat, rabbit†	14 80		**	
Meat, veal†	13 52		**	
Meat, venison†	15 83		**	
Milk, cow's*		2 37		3 44
Milk, cow's†		1 26		1 83
Muskmelon†		7 47		18 82
Oatmeal*	12 93		3 23	
Oatmeal†	10 63		2 66	
Oranges†		5 61		10 94
Peaches†		5 04		12 20
Peanuts†	3 9		0 70	
Peas, dried*		7 07		1 98
Peas, dried†		3 36		0 94
Potatoes I*		7 19		8 63
Potatoes II*		5 5		
Potatoes†		7 72		9 26
Prunes*		24 40		8 05
Prunes†		25 55		8 43
Radishes†		2 87		9 79
Raisins†		23 68		6 87
Raspberry juice†		4 91		
Rice I*	8 1		3 35	
Rice II*	7 08		2 05	
Rice†	8 35		2 42	
Turnips*		2 68		6 86
Turnips†		6 80††		9 41
Wheat, entire*	9 66		3 25	
Wheat, entire†	12 39		3 47	
Wheat, flour*	11 61		2 70	

*Data determined in this laboratory

† Data partly compiled, partly determined in this laboratory

‡ Data published by Katz

** Data insufficient to permit calculation of acid to calorie basis

†† Possible loss of sulphur compounds in drying previous to analysis

calorie basis or on the basis of dry matter. Milk shows a slight predominance of bases. In vegetables and fruits the predominance of bases is usually much greater, a 100 calorie portion of potato for example furnishing enough bases to almost exactly neutralize the excess of acids from a 100 calorie portion of lean beef. The few nuts so far examined yield different results, the peanuts showing an excess of acid-forming elements while the base-forming elements predominate in almonds and chestnuts. It will be of interest to study other edible nuts and to determine whether the partial or complete substitution of nuts for meat produces a marked effect upon the balance of acid-forming and base-forming elements in the diet as a whole.

METABOLISM EXPERIMENTS

In addition to the determination of the balance of acid-forming and base-forming elements in a variety of foods our investigation was planned to include a study of the extent to which the acid arising from oxidation of an "acid-forming" food is neutralized by ammonia when such a food is metabolized in the human body. It was desired to study this point upon a healthy man with ordinary articles of food avoiding any extremes of diet or any unusual condition which might interfere with the normal working of the neutralizing mechanism.

FIRST EXPERIMENT — A healthy man (A O G) twenty-seven years old, 5 feet 7 inches (1.70 meters) high, weighing 142 pounds (64.5 kilograms) took for ten days (November 27 to December 7, 1910) a diet which was uniform throughout except that during the first four and the last two days it contained 340 grams of potato while from the fifth to the eighth day inclusive the potato was replaced by amount of rice (80 grams weighed dry) sufficient to furnish approximately the same energy value (about 300 calories). Since 340 grams of the potatoes here used furnished an excess of base-forming over acid-forming elements equivalent to 15 cc. of normal base while 80 grams of the rice contained an excess of acid-forming elements equivalent to 6.7 cc. of normal acid, the change in diet corresponded to the production in the body of 21.7 cc. of normal acid per day. Beginning with the third day of the experiment the urine was collected in 24 hour

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samples, carefully preserved to prevent ammoniacal fermentation, and the ammonia content of each day's urine was determined by Fohn's method. The total nitrogen of the urine was also determined.

The numerical data of this experiment are briefly summarized in Table 3.

TABLE 3

Average data of first metabolism experiment

INTAKE PER DAY	DIET WITH POTATOES	DIET WITH RICE	DIET WITH POTATOES
	Third and fourth days	Fifth to eighth days	Ninth and tenth days
Lean beef	228 grams	228 grams	228 grams
Butter	142 grams	142 grams	142 grams
Biscuit (dry)	130 grams	130 grams	130 grams
Almonds	60 grams	60 grams	60 grams
Salt	6 grams	6 grams	6 grams
Sugar	50 grams	50 grams	50 grams
Tea	3 cups	3 cups	3 cups
Potato	340 grams		340 grams
Rice		80 grams	
Estimated calories	2780	2773	2780
Estimated excess acid-forming elements in terms of normal solution	11 0 cc	32 7 cc	11 0 cc
OUTPUT IN URINE—AVERAGE PER DAY			
Nitrogen	12 4 grams	10 9 grams	10 3 grams
Ammonia	0 43 grams	0 52 grams	0 43 grams
Ammonia in terms of normal solution	25 1 cc	30 5 cc	25 3 cc

It will be seen that in this experiment a change in diet which increased the excess of acid-forming elements in the daily food by the equivalent of 21 7 cc of a normal solution, caused a rise in the daily ammonia excretion corresponding to only 5 4 cc normal solution. In other words only one-fourth of the extra acid estimated as introduced into metabolism by the change in diet was neutralized by ammonia and eliminated as ammonia salt.

The agreement of results in the first and third periods and the regularity of the data for the individual days (not shown in the condensed table) of the second period make it evident that the relatively small part played by ammonia in the neutralization of the acid cannot be attributed to "lag" in the response of the ammonia metabolism to the change in diet. The response was prompt, both in changing from potato to rice and from rice to potato, but it accounts for only the smaller part of the acid involved.

SECOND EXPERIMENT — The subject and general plan were the same as in the preceding experiment but the analyses were much more detailed. Each of the seven elements concerned in the balance of acids and bases was determined in each article of food and in the urine from each diet, and each day's urine was also analyzed for neutral or unoxidized sulphur, total nitrogen, and total acidity (by the usual method, with phenolphthalein as indicator) as well as for ammonia. This experiment covered two preliminary and nine experimental days, the latter in three consecutive periods of three days each during which the subject maintained a uniform daily schedule as follows. Arose at 7 a m, reached laboratory at 8 30, prepared breakfast, at 9 a m emptied bladder, and began experimental day with first meal, after which did analytical work in laboratory until 1 p m, then took second meal and worked in laboratory till 6 p m, then took third meal and worked again in laboratory until 9 p m, retired at 10 30 p m and slept eight and one-half hours. One liter of water was taken daily in portions of 200 cc at 10 a m, 3, 5, 7 and 9 p m. Somewhat larger amounts of meat, butter and sugar were taken than in the preceding experiment.

The averaged numerical data of this experiment are shown in Table 4.

In this case the change of diet corresponded to an increase of the excess of acid-forming elements equivalent to 28.1 cc normal acid when estimated directly from the amounts of elements in the foods or to 32.7 cc normal acid when allowance is made for the unoxidized sulphur of the urine from each diet. Of this the increased ammonia excretion accounts for 10.7 cc or about one-

TABLE 4
Average data of second metabolism experiment

INTAKE PER DAY	DIET WITH POTATO	DIET WITH RICE	DIET WITH POTATO
	First second and third days	Fourth fifth and sixth days	Seventh eighth and ninth days
Lean beef	270 grams	270 grams	270 grams
Butter	150 grams	150 grams	150 grams
Biscuit (dry)	130 grams	130 grams	130
Almonds	60 grams	60 grams	60 grams
Salt	4 2 grams	4 2 grams	4 2 grams
Sugar	80 grams	80 grams	80 grams
Tea	3 cups	3 cups	3 cups
Potato	340 grams		340 grams
Rice		80 grams	
Estimated calories	3011	3004	3011
Calcium	0 38 grams	0 37 grams	0 38 grams
Magnesium	0 48 grams	0 42 grams	0 48 grams
Sodium	4 79 grams	4 66 grams	4 79 grams
Potassium	3 07 grams	1 66 grams	3 07 grams
Phosphorus	1 22 grams	1 12 grams	1 22 grams
Chlorine	7 55 grams	7 29 grams	7 55 grams
Sulphur	1 25 grams	1 20 grams	1 25 grams
Estimated excess acid- forming elements in terms of normal solu- tion	21 9 cc	52 0 cc	21 9 cc
Excess of acid-forming elements estimated after deducting for un- oxidized sulphur in urine	9 4 cc	42 1 cc	9 4 cc
OUTPUT IN URINE—AVERAGE PER DAY			
Nitrogen	12 9 grams	12 3 grams	12 5 grams
Calcium	0 20 grams	0 22 grams	0 22 grams
Magnesium	0 11 grams	0 11 grams	0 12 grams
Sodium	4 72 grams	3 96 grams	
Potassium	2 32 grams	1 32 grams	
Phosphorus	0 74 grams	0 75 grams	
Chlorine	5 85 grams	5 27 grams	
Sulphur, total	0 94 grams	0 82 grams	0 86 grams
Sulphur, as sulphate	0 74 grams	0 71 grams	0 74 grams
Ammonia	0 41 grams	0 59 grams	*
Ammonia in terms of nor- mal solution	23 8 cc	34 5 cc	*
Acidity in terms of nor- mal solution	21 4 cc	34 1 cc	*

* See table 5

third, while the increased acidity of the urine accounts for 12.7 cc or about two-fifths³

In view of the fact that there has been a tendency to regard increased ammonia output as a test and measure of surplus acid production in the tissues it is interesting to note that in this experiment the increased acidity of the urine played a larger part in the acid elimination than did the increased ammonia output

It is also worthy of note that the total phosphorus of the urine was not increased, indicating that the extra acid produced in metabolism did not, under the conditions of this experiment, have the effect of robbing the body of phosphates. Neither was there any marked change in the output of fixed bases beyond that which may readily be attributed to the differing amounts in the two diets

Some additional points of interest are suggested by an examination of the acidity and ammonia of the urine for the individual days of this experiment as shown in Table 5

It will be seen that on passing from the diet with potato to that with rice the output of ammonia and the acidity of the urine rose immediately, the first day on the rice diet showing essentially the same results as the second and third days, but on changing back from rice to potato the urine did not regain the characteristics of the potato diet until the second day. This may be accidental but if interpreted at face value it would imply that the body responded very quickly to the intake of an acid-forming diet but did not with equal rapidity return to the more normal metabolism when the more normal diet was resumed⁴

³ To determine quantitatively how the body disposed of the acid not accounted for by the increased ammonia and the increased acidity of the urine would lead beyond the scope of this investigation. The possibility of excretion through the intestine is recognized. Analysis of the feces passed on one of the days on each diet showed a difference in balance of acid-forming and base-forming elements which, if accurately representative of the entire periods, would account for most of the excess not accounted for by the ammonia and acidity of the urine. Loss of the feces of the other days through an accident in the laboratory prevented further study of this point. It is possible that a quantitative collection and analysis of the perspiration might also throw light upon the fate of that fraction of the acid produced in metabolism which is not accounted for by the urine

⁴ If the difference in acidity and ammonia between the urines of Febru-

TABLE 5

Total nitrogen, ammonia and acidity of urine of each day in second metabolism experiment

	DATE	TOTAL NITROGEN	AMMONIA	ACIDITY
		grams	grams	cc normal acid
Diet with potato	February 21	12 33	0 39	21 7
	February 22	13 06	0 41	17 7
	February 23	13 17	0 41	24 7
Diet with rice	February 24	13 12	0 58	34 6
	February 25	12 03	0 59	36 7
	February 26	11 77	0 59	31 0
Diet with potato	February 27	11 60	0 49	30 6
	February 28	13 36	0 41	21 8
	March 1	12 64	0 44	30 5

On the last experimental day (March 1, 1911) the division of the food into meals was changed and a part of the meat was eaten at breakfast while all of the potato was eaten at luncheon and dinner. This separation of the principal acid-forming from the principal base-forming food was accompanied by an increased urinary acidity and ammonia output on this day as compared with the preceding day or with the first period when the diet was the same but the meat was always eaten in the same meal with potato. No conclusion should be drawn from the metabolism of a single day but it may not be out of place to suggest that the obvious interpretation of this result (if confirmed by further investigation) would be that in order to obtain the full physiological effect of a balancing of acid-forming and base-forming elements, the foods which contain a marked excess of acid-forming elements should be balanced in each meal by foods in which base-forming elements predominate.

The authors take pleasure in acknowledging their indebtedness to Professor Mandel for the privilege of carrying on a part of the work in his laboratory at the University and Bellevue Hospital Medical College.

ary 27 and 28 be considered as due to the elimination of acid brought into metabolism by the diet of the preceding days, the relative importance of urinary acidity and ammonia output remain unchanged but the total amount of acid accounted for by the urine is somewhat increased and the estimate of the amount otherwise disposed of becomes smaller.

SUMMARY.

The balance of acid-forming and base-forming elements has been estimated from sixty-three ash analyses representing forty-seven different kinds of food, and expressed as surplus acid or base in terms of cubic centimeters of a normal solution per 100 grams and 100 calories of edible material

The meats (including fish) show decided predominance of acid-forming elements. The results are very similar for the lean flesh of different species or of young and mature animals of the same species

The acid-forming elements also predominate in eggs though to a somewhat less degree than in lean meats

When compared on the basis of dry matter or of the 100 calorie portion, grain products show a much smaller predominance of acid-forming elements than do meats and eggs

Milk shows a slight predominance of base-forming elements

Vegetables and fruits show a predominance of base-forming elements, usually much greater than in milk

In two experiments each of several days duration a healthy man took first an ordinary mixed diet containing sufficient potato to furnish about 300 calories or about one-tenth the total value of the diet, then replaced the potato with rice of the same energy value, and later replaced the rice by potato. The change from potato to rice diet involved an alteration of the estimated balance of acid-forming to base-forming elements equivalent to the introduction of 21.7 cc of normal acid per day in the first experiment and 32.7 cc in the second

The ammonia excretion increased in the first case about 21 per cent and in the second about 44 per cent, but this increase was sufficient only to account for one-fourth to one-third of the acid involved

In the second experiment, the acidity of the urine was also determined and the effect of the change of diet noted. The acidity of urine increased about 51 per cent and was found to account for a greater proportion of the acid than was accounted for by the ammonia elimination

In this experiment the increased acidity was not accompanied by any increase in the total phosphorus in the urine

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ON THE ISOLATION OF OOCYTASE, THE FERTILIZING AND CYTOLYZING SUBSTANCE IN MAM- MALIAN BLOOD-SERA

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It has been shown by Loeb¹ that the eggs of sea-urchins (*Strongylocentrotus purpuratus* or *franciscanus*) can be induced to form a fertilization-membrane by immersing them in the sera or tissue extracts of other animals. Their susceptibility to these tissue-extracts varies. Thus if the tissue-extracts or blood of worms, for example, be employed, the eggs of the sea-urchin may be fertilized by simple immersion in the serum without previous treatment. If, however, blood-sera or tissue-extracts of *mammalia* be employed, it is, as a rule, found necessary first of all to sensitize the sea-urchin eggs by previous treatment with a sensitizing agent. The only effective sensitizing agents found by Loeb were exposure to a high temperature (31° to 36°C) or immersion in a solution of strontium or barium chloride (approximately isotonic with sea water). Barium chloride is, however, not practicable to employ for this purpose on account of its high toxicity.

I find that under certain circumstances *calcium chloride* is also capable of acting as a sensitizing agent, although much less powerfully than strontium chloride. On examining various samples of ox-blood serum, one finds a marked variation in their potency as fertilizing or cytolyzing media. Some samples contain

¹ J. Loeb, *Arch f d ges Physiol*, cxviii, p 36, 1907, cxlii, p 96, 1908, cxlii, p 37, 1908. *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, 1909, p 185.

so little of the fertilizing agent² that even after sensitization of the eggs with strontium chloride the proteins present in the serum are sufficient to inhibit the membrane-formation,³ and membranes are only formed in diluted serum (one-eighth to one-sixteenth). Other samples of serum contain more of the fertilizing agent and fertilization-membranes are formed in the *undiluted* serum after sensitization of the eggs with strontium chloride. A few samples of serum contain so much of the fertilizing agent that they will fertilize the eggs of a certain percentage of females without previous sensitization of the eggs. This fact is illustrated by the following experiment.

Freshly centrifugalized α -serum, sample VIII. Rendered isotonic with sea water by the addition of NaCl.

To 2 cc. samples of this serum were added 2 drops each of a thick suspension of the eggs of females A to J of *Strongylocentrotus purpuratus*. The following were the results.

Female A. In eleven minutes 100 per cent fertilized. Marked agglutination.

Female B. In eleven minutes no membranes, in twenty minutes 100 per cent fertilized. The eggs are agglutinated.

Female C. In two hours no membranes, the eggs are, however, agglutinated.

Female D. In two hours only one or two eggs out of some thousands have small "blisters" upon their periphery, none have membranes, and the eggs are not agglutinated.

Female E. None fertilized in thirty-five minutes. No agglutination.

Female F. Less than 1 per cent have membranes in thirty-five minutes. No agglutination.

Female G. In fifteen minutes 100 per cent have membranes and the eggs are agglutinated.

Female H. In fifteen minutes 100 per cent have membranes and the eggs are agglutinated.

Female J. In eight minutes 100 per cent have membranes and the eggs are agglutinated.

Thus out of 9 females 5 were fertilized by this serum and 4 were not. It will be noticed that *the tendency of the eggs to agglutinate or clump together in the serum runs parallel with their susceptibility to the fertilizing action of the serum*.

² The experimental proof of the fact that these variations in potency are due to variations in the oocytase-content of the sera will form the subject of a separate communication.

³ T. Brailsford Robertson, *Univ. of Calif. Pub. Physiol.*, IV, p. 79, 1912.

The eggs of females D, E and F were immersed for four minutes in $\frac{N}{2}$ CaCl_2 and then two drops of each of these samples of eggs were placed in 2 cc of the above serum. The following were the results obtained

Female D Membranes beginning or complete in 100 per cent in five minutes. The eggs are agglutinated

Female E In four minutes the eggs are agglutinated and over 50 per cent have membranes. In ten minutes there were membranes on 100 per cent

Female F In three minutes over 50 per cent have membranes and in ten minutes 100 per cent. The eggs are agglutinated

It is therefore clear (1) *that under favorable circumstances, i. e., if the fertilizing agent be abundant in the serum, calcium chloride sensitizes the eggs of sea-urchins to the fertilizing agent. The action is therefore one which is common to all of the alkaline earths,* (2) *that the same agent which sensitizes for fertilization also sensitizes for agglutination*

I observed that on adding BaCl_2 or SrCl_2 to normal serum, or CaCl_2 to heated serum (57° for three hours, which does not destroy the fertilizing agent⁴), a precipitate is produced (in the case of BaCl_2) or an opalescence (SrCl_2 or CaCl_2) which does not immediately disappear on rendering the serum acid. It occurred to me, therefore, that the fertilizing agent might be precipitable by alkaline earths and that the sensitizing action of the alkaline earths might be due to the fact that they precipitate the fertilizing agent upon the egg. This possibility indicated a procedure for isolating the fertilizing agent from blood-sera which, after several trials, finally enabled me to obtain a preparation of the fertilizing agent which is so potent that it exerts a marked action upon sensitized eggs (SrCl_2 sensitization) at a dilution of one to twenty-five thousand.

It has been observed by Loeb that the fertilizing agent is precipitated by acetone. I accordingly proceeded as follows

To 860 cc of the isotonic serum (sample VIII) alluded to above, I added 400 cc of 7 per cent BaCl_2 . A thick cloud is produced. On standing in a warm place (37°) for an hour a fine precipitate settles upon the bottom of the vessel, which, on gently agitating, clumps together in coarse heavy flocculi. Barium carbonate and sulphate do not settle so quickly as this in solutions containing

⁴ Loeb *Loc cit*

colloids, and the supernatant cloud which still remains consists in the main, probably, of these substances. Nevertheless in order to be sure of obtaining a tolerably complete yield, I centrifuged the mixture. The entire precipitate settles readily in the form of a thick cake at the bottom of the centrifuge tubes. This precipitate is thoroughly drained and then is shaken up in 2 per cent BaCl_2 several times and re-centrifuged to rid it of adherent serum. The washed precipitate from the entire 860 cc of serum was now suspended in 100 cc of $\frac{N}{10}$ HCl and stirred continuously for one half hour until the residual undissolved material (probably BaSO_4) was thoroughly broken up into a fine powder.

The yellowish clear solution obtained by centrifugalizing this mixture contains the fertilizing agent and barium chloride. In order to free it from BaCl_2 I added 10 cc of 10 per cent Na_2SO_4 solution and allowed the mixture to stand at 50° over night. The mixture was then centrifuged and the clear yellowish supernatant fluid tested for barium by the addition of Na_2SO_4 . It yielded no precipitate or opalescence and was therefore free from barium.

To this fluid were added four volumes of acetone. A light flocculent precipitate was formed at once, which settled readily. This was collected upon a hardened filter, washed twice with 500 cc of alcohol and twice in 500 cc of ether⁵ and dried over H_2SO_4 at 36° for twelve hours. The substance was thus obtained in the form of a pure white, slightly caked powder. This was pulverized and further dried over H_2SO_4 for three days at 36°C .

This substance, of which about 150 mgs in all were obtained, dissolved only in traces in sea water, but it dissolved readily and quickly in $\frac{N}{10}$ HCl . Accordingly, 80 mgs were dissolved in 6.5 cc of $\frac{N}{10}$ HCl and the clear yellowish solution thus obtained was exactly neutralized by the addition of 6.5 cc of $\frac{N}{10}$ NaOH . The solution became somewhat opalescent. This solution was now rendered isotonic with sea water by the addition of 2.2 cc of $\frac{5M}{2}$ NaCl , and then diluted to one, one-half, one-fourth, and so forth by the addition of sea water, forming solutions containing 1 part in 200, 400, and so forth of the fertilizing agent. On adding sea water the opalescence of the solution greatly increased, the mixture containing an equal volume of sea water and of the original solution being

⁵ These washings were carried out inside an incubator over sulphuric acid in order to avoid the deposition of atmospheric moisture upon the filter.

almost milky This opalescence continued to be very marked down to a dilution of one-sixty-fourth

The eggs of one female *Strongylocentrotus purpuratus* were divided into three portions One portion was not sensitized at all, another was sensitized by four minutes' immersion in $\frac{x}{2}$ SrCl_2 , and a third by 4 minutes' immersion in $\frac{x}{2}$ CaCl_2 Two drops of thick egg-suspension were added to 2 cc of each of the dilutions of the solution just described The following were the results

Eggs Sensitized with SrCl_2

Dilution of the solution
of the fertilizing agent

1 part in 200

Effect of immersing the eggs in this solution

Immediate agglutination * In fifteen minutes irregular crinkled and collapsed membranes surround the majority of the eggs

1 part in 400

1 part in 800

1 part in 1,600

1 part in 3,200

1 part in 6,400

Immediate agglutination A dense precipitate surrounds the agglutinated masses of the eggs so that the individual eggs cannot be discerned

Immediate agglutination A dense precipitate surrounds each egg After two hours in all those cases in which the egg itself can be observed there is a distinct membrane

1 part in 12,800

Agglutinated in one minute Blisters on several of the eggs in four minutes No change after seventy-five minutes

1 part in 25,600

Slight agglutination in one minute No further change in seventy-five minutes

1 part in 51,200

No agglutination From this dilution down to that of 1 part in 409,600 the substance had no action upon the eggs

* This agglutination is not to be confused with the phenomenon of "stickiness" which is exhibited by all eggs which have been treated with strontium chloride When eggs which have been treated with strontium chloride are dropped into sea water they soon sink to the bottom of the vessel and adhere to it in a thin layer, even if shaken up before they sink, they do not adhere in clumps, at most one or two sticking together very loosely so that they can readily be shaken apart again Eggs which "agglutinate," in the sense in which the word is used above, "clot" or form large clumps resembling coagula almost the instant they are dropped into the agglutinating mixture

Unsensitized Eggs

1 part in 200	Irregular membranes and blisters and agglutination within one minute In four minutes 10 per cent of the eggs are cytolyzed In five minutes crinkled and collapsed membranes upon all of the eggs In thirty minutes 50 per cent cytolyzed or converted into "shadows"
1 part in 400	Irregular blisters on 20 per cent of the eggs in five minutes Agglutination occurred at once Irregular crinkled membranes in about 50 per cent in fifteen minutes
1 part in 800	Agglutination occurred at once In fifteen minutes 100 per cent have perfect spherical membranes
1 part in 1,600	No agglutination From this dilution down to that of 1 part in 409,600 the substance had no action upon the eggs

Eggs Sensitized with CaCl_2

1 part in 200	Blisters and irregular crinkled and collapsed membranes and pronounced agglutination within one minute In fifteen minutes 10 per cent cytolyzed In two hours 80 per cent cytolyzed
1 part in 400	Pronounced agglutination in one minute In two hours blisters upon all of the eggs but no complete membranes
1 part in 800	Agglutinated in one minute In five minutes large and perfectly spherical membranes on 100 per cent, each membrane having a fine precipitate imbedded in it here and there upon the periphery
1 part in 1,600	Agglutinated in one minute In two hours a few have membranes but the number cannot be clearly made out owing to the flocculent precipitate which surrounds the eggs
1 part in 3,200	No agglutination From this dilution down to that of 1 part in 6,400 the substance had no action upon the eggs

From the results it is clear that a substance precipitable from serum by BaCl_2 or by acetone and soluble in dilute acids and salt solutions is capable of bringing about membrane formation, partial or complete, at certain dilutions. The eggs can also be sensitized to the action of this substance by previous immersion in solutions of SrCl_2 and CaCl_2 and the sensitizing action of these substances is clearly seen to reside in their power of forming an insoluble compound with the fertilizing agent and precipitating it upon the eggs. So dense is this precipitate when SrCl_2 is employed as the sensitizing agent that if membranes are formed in solutions of dilutions of from 1 part in 400 to 1 part in 3200 they cannot be observed because the precipitate completely envelops the eggs and hides them from view.

The relative sensitizing powers of CaCl_2 and SrCl_2 can readily be compared from the following summary of results enumerated above.

Unsensitized eggs	1 part in 800
Eggs sensitized with CaCl_2	1 part in 1,600
Eggs sensitized with SrCl_2	1 part in 25,600

It will be recollected (cf. above) that the power of serum to agglutinate sea-urchin eggs runs parallel with its power to fertilize them and that SrCl_2 and CaCl_2 sensitize the eggs to both processes.

It would appear highly probable, therefore, that the substance which can be isolated from active sera by the process outlined above is the substance which is responsible for the fertilizing, cytolyzing and agglutinating action of these sera upon sea-urchin eggs.

Prior to the experiment reported in detail above I made several impure preparations of the substance, contaminated with BaSO_4 , proteins, etc., all of which fertilized and agglutinated sea-urchin eggs after sensitization with SrCl_2 .

The small amounts of this substance which I have as yet been able to obtain have not sufficed to carry out any extensive investigations upon its chemical properties. The preparations which I have made, however, yield Millon's test.

From data which I have obtained and which will be reported subsequently, it appears probable that the fertilizing agent is not present as such in circulating blood, but is derived from the

breaking down of corpuscles in shed blood. The fertilizing agent is also thermostable, resisting an exposure of nineteen hours to a temperature of 58°. It consequently appears to be analogous to the cytases or cell-liquefying substances observed by Metchnikoff⁷ and others to be derivable from white corpuscles. I therefore suggest that this substance be termed *oocytase*.

⁷ E. Metchnikoff *L'Immunité dans les maladies infectieuses*, Paris, 1902

ON THE COMBINED ACTION OF MUSCLE PLASMA AND PANCREAS EXTRACT ON SOME MONO- AND DISACCHARIDES

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Through the experiments reported in a previous communication¹ the present writers have demonstrated that by the combined action of muscle plasma and pancreatic extract *d*-glucose was converted into a disaccharide. On the other hand, under the same conditions of experiment maltose was cleaved into glucose. Naturally it became important to make clear whether the action was applicable also to other sugars. Of the previous writers only Hall² allowed the muscle plasma to act on other sugars than glucose. This investigator extended his experiments to *d*-levulose, *l*-arabinose and *d*-xylose, and was led to the conclusion that the glycolytic action of his enzyme mixture was limited to glucose only. In the course of our previous work the optimal conditions for the action of muscle plasma and pancreatic extract were determined with greater certainty, and this made it urgent to repeat and to extend the experiments of Hall, all the more since the action of the enzyme mixture is viewed at present in a different light.

Of the hexoses levulose, mannose and galactose were employed. The mannose was obtained through the courtesy of Dr. Hudson of Washington, and we wish to express our appreciation for his kindness. Of the pentoses *l*-arabinose, *d*-xylose and *d*-ribose were used for the experiments. Lactose was the disaccharide tested.

In regard to hexoses it was found that mannose remained unchanged under the conditions of our experiments, but *d*-levulose showed under the influence of muscle plasma and of pancreatic

¹ Levene and Meyer, this *Journal*, **14**, pp 97-107, 1911.

² Hall, *Amer Journ of Physiol*, **xviii**, pp 283-294, 1907.

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extract a diminution of reducing power which could be restored to nearly the original power by means of hydrolysis with dilute mineral acid

In this respect our conclusions differ from those of Hall. The analysis of tables published by Hall, however, shows some disappearance of levulose through the action of the enzyme mixture, but the writer is inclined to explain these changes by faults in technique, namely by bacterial contamination. On the other hand, there is no record in his report of the concentration of the sugar employed in his experiments, and the importance of this factor has been emphasized in our previous publication. All our experiments were controlled by bacteriological examination, and only those experiments were taken into consideration which proved free from any bacterial growth. Hence the disagreement between our results and those of Hall in regard to *d*-levulose is probably caused by the difference in the sugar concentration employed in the experiments of Hall and in ours.

Regarding the pentoses our experiments are in full accord with those of Hall, and there was never observed a diminution in the reducing power of the pentose even when the concentration of the sugar solutions employed in the experiments was very considerable.

Also in regard to lactose the results of our experiment harmonize with those of Hall.

On the basis of all this experience one is led to the conclusion that the muscle plasma combined with pancreatic extract possesses the power to cause condensation of only two closely related hexoses, namely of *d*-glucose and of *d*-levulose, and that it remains without action on mannose, xylose, ribose, and lactose. The same enzyme mixture also has the power to bring about the hydrolysis of maltose, but not of lactose.

EXPERIMENTAL PART

The enzyme mixtures were prepared in the manner described in the previous communication.

All the details of sugar analysis were the same as there described.

The condensed levulose was hydrolyzed by heating on a water bath for two hours with 5 per cent hydrochloric acid.

The results of the analysis are given in the following tables

d-Levulose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NHCNS	NHCNS PER CUBIC CENTIMETER	LAEVULOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS PER 100 CUBIC CENTI- METERS	PERCENTAGE LOSS
<i>a</i> At beginning of experiment	0 5	28 7	57 4	20 09		
After thirty-six hours	0 5	25 8	51 6	18 06	2 03	10 1
<i>b</i> At beginning of experiment	0 5	24 1	48 2	17 2		
After thirty-six hours	0 5	22 4	44 8	16 0	1 2	7 0
After hydrolysis	0 5	23 2	46 4	16 6		
<i>c</i> At beginning of experiment	1 0	27 9	27 9	10 0		
After thirty-six hours	1 0	26 3	26 3	9 4	0 6	6 0
<i>d</i> At beginning of experiment	2 0	31 2	15 6	5 46		
After thirty-six hours	2 0	29 4	14 6	5 21	0 25	4 5

Galactose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NHCNS	NHCNS PER CUBIC CENTIMETER	GALACTOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS
<i>a</i> At beginning of experiment	0 5	23 55	47 1	17 23	
After thirty-six hours	0 5	23 60	47 2	17 28	0
<i>b</i> At beginning of experiment	0 5	19 0	38 0	13 93	
After thirty-six hours	0 5	19 1	38 2	13 95	0
<i>c</i> At beginning of experiment	1 0	22 8	22 8	8 34	
After thirty-six hours	1 0	22 6	22 6	8 34	0
<i>d</i> At beginning of experiment	2 0	22 9	11 45	4 17	
After thirty-six hours	2 0	22 9	11 45	4 17	0

l-Arabinose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NHCNS	NHCNS PER CUBIC CENTIMETER	ARABINOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS
<i>a</i> At beginning of experiment	0 5	26 4	52 8	14 8	
After thirty-six hours	0 5	26 4	52 8	14 8	0
<i>b</i> At beginning of experiment	1 0	21 6	21 6	6 37	
After thirty-six hours	1 0	21 6	21 6	6 37	0

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d-Xylose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NH ₄ CNS	NH ₄ CNS PER CUBIC CENTIMETER	XYLOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS
<i>a</i> At beginning of experiment	0 5	24 1	48 2	12 35	
After thirty-six hours	0 5	24 1	48 2	12 35	0
<i>b</i> At beginning of experiment	1 0	24 3	24 3	6 22	
After thirty-six hours	1 0	24 3	24 3	6 22	0

Lactose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NH ₄ CNS	NH ₄ CNS PER CUBIC CENTIMETER	LACTOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS
<i>a</i> At beginning of experiment	3 0	18 6	6 20	12 0	
After thirty-six hours	3 0	18 5	6 17	12 0	0
<i>b</i> At beginning of experiment	4 0	17 0	4 25	8 0	
After thirty-six hours	4 0	17 2	4 30	8 0	0

d-Ribose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NH ₄ CNS	NH ₄ CNS PER CUBIC CENTIMETER	<i>d</i> -RIBOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS
At beginning of experiment	0 5	22 2	44 4	15 8	
After thirty-six hours	0 5	22 1	44 2	15 8	0

Mannose

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NH ₄ CNS	NH ₄ CNS PER CUBIC CENTIMETER	MANNOSE, GRAMS PER 100 CUBIC CENTIMETERS	LOSS
<i>a</i> At beginning of experiment	0 5	24 4	48 8	15 05	
After thirty-six hours	0 5	24 2	48 4	15 05	0
<i>c</i> At beginning of experiment	1 25	31 8	25 44	7 84	
After thirty-six hours	1 25	31 7	25 4	7 84	0
<i>b</i> At beginning of experiment	1 0	36 5	36 5	11 25	
After thirty-six hours	1 0	36 5	36 5	11 25	0

In calculating the grams sugar for 100 cc solution the following values for 1 cc $\frac{N}{10}$ NH₄CNS equivalent to milligrams sugar were used

	<i>Mgs</i>		<i>Mgs</i>
Glucose	3 58	Arabinose	2 95
Laevulose	3 58	Xylose	2 56
Maltose	5 78	Ribose	2 56
Galactose	5 76	Lactose	1 89

ON THE ACTION OF VARIOUS TISSUES AND TISSUE JUICES ON GLUCOSE

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The literature on the glycolytic action of various animal organs contains most contradictory and confusing statements. While some writers claimed the presence of sugar-destroying enzymes in all organs and tissues, other observers detected such enzymes only in few organs, and only under very definite conditions, namely, in the presence of some auxiliary substance. Thus, in recent years, Arnheim and Rosenbaum¹ and Stoklasa² and his co-workers claimed a general distribution of sugar-destroying enzymes in all animal tissues. Rapoport³ observed glycolytic action only in blood and fibrin and obtained negative results from his experiments with other organs. Finally, R. Hirsch⁴ and O. Cohnheim⁵ observed that the glycolytic action is brought about by the combined action of pancreas extract and of the liver, or by pancreas extract and muscle plasma.

It is possible that the observations reported by every one of the writers are correct, and the apparent contradictions were brought about by the different conditions of the respective experiments. It became evident from our work on the combined action of muscle plasma and pancreatic extract that alone variations in the sugar concentration may change the results of the experiments to such an extent that a marked disappearance of glucose will

¹ *Zeitschr f physiol Chem*, xl, p 220, 1903-1904

² *Pflüger's Archiv*, ci, p 311, 1904

³ *Zeitschr f Klin Med*, lvi, p 208, 1905

⁴ *Hofmeister's Beiträge*, iv, p 535, 1903

⁵ *Zeitschr f physiol Chem*, xxxix, p 336, 1903, xlii, p 401, 1904, xliii, p 547, 1904-1905, xlvi, p 253, 1906

take place in one instance, and no change in the sugar content in the other. An analogous observation was made in the work on leucocytes, which will be reported subsequently.

Another factor determining the result of the experiment lies in the degree of alkalinity or acidity of the solutions used in the experiment. The importance of this factor was pointed out first by the work of Hall, and was corroborated by our own experiments. In agreement with Hall we found that Henderson's phosphate mixture offers the best medium for the study of the so-called glycolytic process. Still another cause for divergence in the characteristics of the results one may find in the difference in the species of the animal whose organs were employed in the experiment. Recent work on the enzymes of animal tissues has brought to light the great differences in the enzyme content of analogous organs of animals belonging to different species. Of course some of the discrepancies in the results of individual writers may have been caused by the different degrees of antiseptic precaution exercised by them. Only rarely were experiments controlled by bacteriological examinations. Further, the analytical methods employed by different workers varied greatly in their accuracy. And yet another source for possible error may be found in the fact that rarely was there made an attempt to search for the products of the disappearing sugar.

The knowledge of the products formed in course of the experiment is important not only for theoretical reasons but as a means of detecting bacterial contamination. Thus in all our experiments of the last two years carbon dioxide was only rarely detected in the reaction mixture, and its presence always indicated bacterial contamination, therefore, we are inclined to believe that in the experiments of other writers, where there was reported the formation of carbon dioxide from sugar, this resulted from bacterial activity, and not through the actions of tissue enzymes.

All these considerations led us to subject to a revision all the older observations on the presence in animal organs of either "glycolytic" enzymes or of activators of the enzymes, all the more since it became evident that the so-called glycolysis under the combined action of muscle plasma and pancreas consisted in a condensation and not in a destruction of glucose. In course of this

work the organs of the rabbit and of the dog were employed In one series of experiments the enzymotic effect of the tissue pulp was tested and in another of the tissue juices A still different series of experiments aimed to investigate the presence in various organs of enzyme activators For this purpose the action of various organs aided by extracts of other organs was tested Every experiment was controlled by bacteriological examinations, made by Dr Bronfenbrenner, only experiments that proved free from any bacterial growth were taken into consideration

The results of the experiments were as follows

A In the experiments with the rabbit, without the aid of activators only the action of liver and of muscle tissues were tested The results in both experiments were negative With the aid of pancreas extract also only the same two tissues were tested Positive results were obtained only with the muscle plasma

B In the experiments with the dog without the aid of activators the following tissue juices were employed muscle, lung, intestine, kidney, pancreas and spleen All experiments were negative

As activators the extracts of the pancreas and of the spleen were employed With each activator were employed the same tissue juices as in the first series The results were the following The addition of pancreas extract did not alter the action of the tissue juices, on the other hand, after the addition of spleen extract as activator there was observed a fall of the reducing power of the sugar solution in the experiments with muscle, lung, liver and pancreas The action was of very moderate intensity

In the experiments with tissue pulp the following organs were used muscle, spleen, liver, lung and pancreas The results in all experiments were negative, excepting the liver The experiments with liver tissue and additional glucose showed at the end of the experiments no change in the original reducing power, on the other hand, in the control experiments with liver tissue alone there was observed an increase in the reducing power Hence it follows that in the experiment with additional glucose there was a compensation of phenomena so that the rise of glucose formation was observed by a simultaneous disappearance of glucose

With the addition of pancreas extract as activator the same organs were used The results were the same as in previous series

With the addition of spleen extract as activator the presence of enzymotic action was observed in the experiments with muscle, lung, liver and pancreas tissues

Thus in the dog the spleen and not pancreas is the organ containing the activator for the enzyme causing the condensation of glucose

The general conclusion from the present experiments is that under the conditions here reported, namely, in the presence of antiseptics and under the conditions where access of oxygen is not totally excluded, animal tissues or their juices, aided or unaided by auxiliary substance fail to bring about a destruction of glucose. Wherever a fall in the reducing power of a sugar solution was brought about by the combined action of tissue and activator this was due to a condensation of the glucose. However, the writers realize that under some other conditions an actual glycolysis may take place and it is hoped the exact conditions will be determined at some future date

EXPERIMENTAL PART

The animals used in the experiments were killed by bleeding from the jugular vein. The organs were removed under aseptic conditions and immediately employed for the preparation of either plasma or tissue pulp

Tissue Plasma For the preparation of this, the organs were hashed and extracted for several hours with the Henderson's phosphate mixture, and then strained through cheese cloth, the residue was ground with sand and pressed in a Buchner press at 300 atmospheres. All the liquids were combined and employed for the experiments

Tissue Pulp Five grams of the freshly prepared organ pulp were added to a flask containing 45 cc of a solution of glucose in Henderson's phosphate solution

Intestinal Extract was prepared in the following way. The intestines were washed and the mucous membrane scraped off with a knife. The substance was taken up in a Henderson's phosphate solution containing glucose and well agitated

Activators Ten grams of the hashed organ were treated in identically the same manner as previously described for the pancreas

The final solution was made up to 10 cc and 1 cc added to each flask of 50 cc

Sugar Estimations The reducing power was determined in all cases on the strained mixture Ten cubic centimeters of this liquid were coagulated by boiling and the addition of acetic acid, and made up to 100 cc without filtering This solution was filtered through a dry filter and five, ten or more cubic centimeters used for each reduction The reduced copper was determined by the Volhard method

Organs of a rabbit

A rabbit was starved for three days and placed in a room below freezing temperature for three hours prior to its execution

	TISSUE	ACTIVATOR	NH ₄ CNS	NH ₄ CNS PER CUBIC CENTI METER	GLUCOSE GRAMS PER 100 CUBIC CENTIMETERS	100g PER CENT
Before	Muscle	None	20 0	40 0	15 32	12 5
After			20 0	40 0		
Before	Muscle	Pancreas	24 0	48 0	17 18	
After			21 0	42 0	15 03	
Hydrolyzed			23 6	47 2	16 90	
Before	Liver	None	23 5	47 0	16 82	
After			23 5	47 0		
Before	Liver	Pancreas	22 9	45 8	16 39	
After			23 1	46 2	16 53	

1 cc liver plasma—no reduction

1 cc liver plasma hydrolyzed—no reduction

Tissue plasma of dog

		TISSUE	ACTIVATOR	NH ₄ CNS	NH ₄ CNS PER CUBIC CENTI- METER	GLUCOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS PER 100 CUBIC CENTI- METERS	LOSS PER CNT
				cc				
Before	}	Muscle	None	20 6	41 2	14 55		
After				20 6				
Before	}	Muscle	Spleen	23 0	46 0	16 5		
After				21 9	43 8	15 6	0 9	4 8
Hydrolyzed	}	Muscle	Pancreas	22 3	44 6	16 0		
Before				21 6	43 2	15 5		
After	}	Muscle	Spleen	21 5	43 0			
Before				27 0	54 0	19 3		
After	}	Muscle	Pancreas	25 2	50 4	18 06	1 24	6 4
Before				30 0	60 0	21 5		
After	}	Lung	None	30 0	60 0	21 5		
Before				22 0	44 0	15 75		
After	}	Lung	Spleen	21 7	43 4	15 55		
Before				20 1	40 2	14 3		
After	}	Lung	Pancreas	16 9	33 8	12 05	2 25	15 7
Hydrolyzed				19 0	38 0	13 65		
Before	}	Lung	Spleen	20 1	40 2	14 4		
After				20 0	40 0			
Before	}	Lung	Spleen	28 5	57 0	20 4		
After				27 5	55 0	19 69	0 71	3 5
Before	}	Intestine	None	22 5	45 0	16 08		
After				22 8	45 6	16 30		
Before	}	Intestine	Spleen	20 6	41 2	14 75		
After				20 8	41 6	14 89		
Before	}	Intestine	Pancreas	20 4	40 8	14 60		
After				20 4	40 8			
Before	}	Intestine	Pancreas	33 0	66 0	23 62		
After				33 1	66 2			
Before	}	Intestine	Spleen	32 6	65 2	23 32		
After				32 8	65 6	23 48		
Before	}	Kidney	Spleen	26 6	53 2	19 04		
After				26 8	53 6	19 18		
Before	}	Pancreas	Spleen	29 6	59 2	21 19		
After				29 5	59 0			
Before	}	Spleen	Pancreas	32 3	64 6	23 12		
After				32 2	64 4			

Organ pulp of dog, without activator

	TISSUE	NH ₄ CNS	NH ₄ CNS PER CUBIC CENTI METER	GLUCOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS PER 100 CUBIC CENTI METERS	LOSS PER CENT
Before	Muscle	16.9	33.8	12.10	0.14	1.0
After		17.0	34.0			
Before	Spleen	23.7	47.4	16.96		
After		23.7	47.4			
Before	Liver	19.2	38.4	13.74		
After		19.0	38.0	13.60		
Before	Pancreas	25.4	50.8	18.18		
After		25.5	51.0			
Before	Lung	23.7	47.4	16.96		
After		23.6	47.2			

Organ pulp of dog, with activators

	TISSUE	ACTIVATOR	NH ₄ CNS PER CUBIC CENTI METER	GLUCOSE GRAMS PER 100 CUBIC CENTIMETERS	LOSS PER 100 CUBIC CENTI METERS	LOSS PER CENT
Before	Muscle	Spleen	40.4	14.46	0.43	2.9
After			39.2	14.03		
Before	Lung	Spleen	34.4	12.31	0.21	1.7
After			33.8	12.10		
Before	Liver	Pancreas	35.4	12.70	0.00	
After			35.4	12.70		
Before	Liver	Spleen	34.2	12.24	0.14	1.1
After			33.8	12.10		
Before	Pancreas	Spleen	32.0	11.45	0.36	3.1
After			31.0	11.09		

Organ pulp of dog, without glucose

5 grams muscle pulp	No reduction
5 grams spleen pulp	No reduction
5 grams pancreas pulp	No reduction
5 grams lung pulp	No reduction
5 grams	
liver pulp { Before	10-9.3-0.7 NH ₄ CNS = 0.25 per cent
{ Hydrolyzed	10-7.9-2.1 NH ₄ CNS = 0.75 per cent

THE ACTION OF LEUCOCYTES ON GLUCOSE

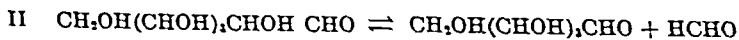
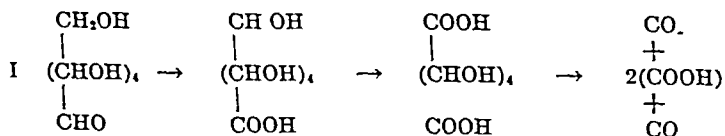
BY P A LEVENE AND G M MEYER

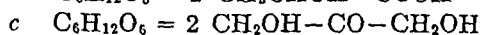
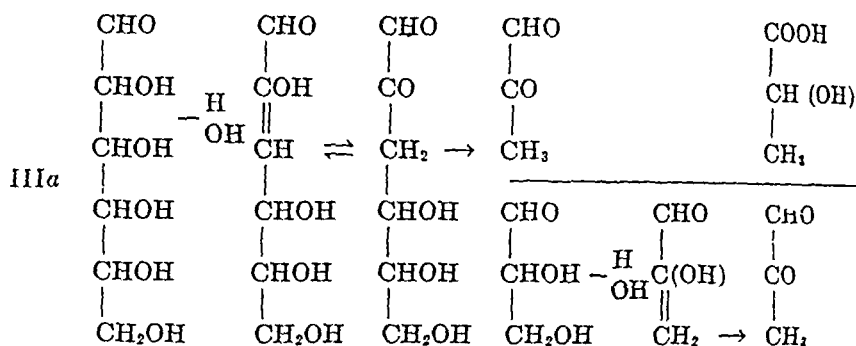
(From the Rockefeller Institute for Medical Research, New York)

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The final products of sugar combustion in the animal organism are carbonic acid and water. This information is undisputable. The knowledge of every other phase in the complex process of glycolysis has not advanced beyond the state of conjecture. The paucity of undisputed knowledge regarding this very important biological process is due to the fact that intravital reactions are made visible only with great difficulty, and secondly to the great variety of theoretically possible processes which may lead to the breaking of the links between the individual carbon atoms of the sugar molecule.

There are three principal types of sugar degradations. One begins directly with the oxidation of the end carbon atoms, the second consists in a gradual dissociation of formaldehyde. It represents a reversion of the synthesis of sugar from formaldehyde. The first step of this reaction leads to the formation of pentose from glucose. The reaction of the third type leads to a splitting of the six-carbon chains of glucose into two three-carbon chains—with the formation of either dioxyacetone or of lactic acid as the first phase of the reaction.





Each one of the three types of reactions was taken into consideration for the explanation of the process of sugar combustion in the body and attempts were made to interpret the process on the basis of every one of the three reactions

The method employed for the purpose of bringing to light the mechanism of biological cleavage of sugar were the following

1 The search in the tissues for substances that may originate from carbohydrates

2 Experiments on the action of tissues and tissue extracts on sugar

3 Perfusion of organs with carbohydrates and their cleavage products

4 Feeding healthy and diabetic animals on carbohydrates and their oxidation product

None of the methods has furnished thus far convincing evidence which would permit the acceptance, or would force the rejection of either one of the possible interpretations of sugar combustion in the animal. For the reasons which will become evident later, we shall briefly review the work which aimed to analyze the possibility of the intermediate cleavage of hexose into two molecules each containing a chain of three carbons. Four substances had been named in connection with the biological sugar combustion: lactic acid, glyceric aldehyde, methylglyoxal and dioxyacetone.

In connection with sugar combustion in the animal organism only lactic acid received serious consideration. The formation of lactic acid in surviving tissues at the expense of disappearing glycogen was accepted by older writers. In those experiments the

influence of bacteria was not excluded, and the subject received a revision in recent years. The views of the writers who were actively engaged in the investigation of lactic acid in the animal organism are equally divided, some regarding protein and others carbohydrates as the source of the substance. The writers who in recent years contributed evidence in support of the carbohydrate origin of lactic acid were Spiro,¹ Hoppe-Seyler² and his co-workers, Araki³ and Zillesen,⁴ Embden,⁵ and particularly Graham Lusk and A R Mandel.⁶ The most emphatic partisan of the protein origin of lactic acid was Minkowski⁷ and his views are supported by evidence adduced by Asher and Jackson,⁸ and by Neuberg and Langstein.⁹

The views of Hoppe-Seyler and his co-workers were based on observations on animals placed in conditions which brought about insufficient oxygenation of the tissues. In all such conditions there was observed the elimination of lactic acid through the urine. Inouye and Saiki made similar observations in epilepsy. Embden reached his conclusions on the basis of perfusion experiments. The perfusion of livers rich in glycogen resulted in lactic acid formation. The same result was observed when blood containing sugar was perfused through a liver poor in glycogen. On the other hand, perfusion of a liver freed from glycogen with blood of a very low sugar content failed to bring about lactic acid formation. Lusk and Mandel based their view on experiments on dogs which received combined phloridzin and phosphorus treatment. It is known that phosphorus injected into normal animals causes the elimination of lactic acid. On the other hand, the injection of phloridzin caused the removal, through the urine, of glucose from the body tissues. If the phosphorus injection was preceded by a phloridzin injection it failed to give rise to an elimination of lactic acid. The

¹ *Zeitschr f physiol Chem*, 1, p 111, 1877

² *Festschr zu Virchow's Jubiläum*

³ *Zeitschr f physiol Chem*, xv, pp 335 and 546, 1891, xvi, pp 201 and 453, 1892, xvii, p 311, 1893, xix, p 422, 1894

⁴ *Zeitschr f physiol Chem*, xv, p 387, 1891

⁵ *Centralblatt f Physiol*, xviii, p 832, 1905

⁶ *Amer Journ of Physiol*, xvi, p 129, 1906

⁷ *Arch f exp Path u Pharm*, xvi, p 67, 1886, xxxi, p 214, 1893

⁸ *Zeitschr f Biol*, xli, p 393, 1901

⁹ *Arch f Physiol*, Suppl 1903, p 514

interpretation given by Lusk and Mandel to these observations was that normally under the influence of phosphorus lactic acid is formed at the expense of glucose, and that phloridzin in removing glucose also removes the mother substance of lactic acid

On the other hand, Minkowski observed that birds, after removal of the liver, eliminate through their urine considerable quantities of lactic acid, and that its value is influenced by the protein intake and not by that of carbohydrates Jackson and Asher in perfusion experiments failed to detect any influence of carbohydrate on the lactic acid formation and Neuberg and Langstein demonstrated the appearance of lactic acid in the urine after administration of alanine Thus the evidence in support of either view was mostly indirect and not sufficiently convincing These considerations impelled us a year ago to undertake the study of the products formed in course of glycolysis by means of tissue extracts The results were all negative For various reasons it was deemed advisable to test the influence on glucose of living leucocytes Lepine¹⁰ and his co-workers and Mayer¹¹ had already advanced the view that leucocytes were concerned in the process of glycolysis Their evidence was indirect and the products of leucocytic glycolysis remained unexplained by them

In the present investigation all the experiments were performed under absolutely aseptic conditions The solutions were always tested for aerobic and anaerobic microorganisms by Dr Bronfenbrenner to whom we are greatly indebted for that part of the work The leucocytes were suspended in a sugar solution containing 15 per cent of the Henderson phosphate mixture The results of the experiments were the following

- 1 Under the influence of leucocytes a sugar solution loses part of its reducing power

- 2 The reducing power cannot be restored to the original by boiling with mineral acids

- 3 The rate of glycolysis is in inverse proportion to the sugar concentration (The last two points are interesting in connection with the influence of muscle plasma and pancreatic extract of glucose There the fall in the reducing power was in direct pro-

¹⁰ *Le diabète sucré*, Paris, 1909

¹¹ *Arch de Physiol* 2

portion to the sugar concentration, and the original reducing power could be restored by hydrolysis with mineral acids)

4 If distilled water is employed in place of the phosphate mixture the leucocytes fail to exert any influence on glucose

5 If toluol is added to the phosphate mixture the leucocytes do not demonstrate any action on glucose

6 As product of the action of leucocytes on glucose, paralactic acid was discovered It was identified as the zinc salt Volatile acids were not detected

7 The quantity of lactic acid found was lower than that of the disappeared glucose Whether the missing sugar underwent decomposition into other substances than lactic acid, or was used for synthetic purposes by the leucocytes remains to be established

EXPERIMENTAL

Leucocytes Medium sized dogs were given two injections of 1.5 cc turpentine into the pleural cavity at an interval of three days Ether narcosis was used at the first injection Eighteen hours after the second injection the liquid which had formed in the pleural cavity was withdrawn by aspiration This exudate, which contained the greater portion of the turpentine, was discarded The following day the aspiration was repeated This fluid was received into sterile bottles The color of the exudate was a straw yellow occasionally tinged red The quantity of exudate obtained from each dog varied greatly Three or more dogs were injected simultaneously so as to insure an ample supply of leucocytes The combined exudate was centrifugalized and the leucocytes washed twice with sterile physiological saline The centrifugal flasks contained glass beads to facilitate the breaking up of the agglutinated mass and thus aid proper washing and mixing

Solutions In the first three experiments the leucocytes were well shaken with sterilized 1 per cent Henderson phosphate solution and this suspension added to flasks containing the desired quantity of glucose Merck's "highest purity" glucose was used in all experiments Just enough water was added previous to sterilizing, so that it remained liquid after cooling In later experiments, where only one concentration of glucose was desired,

the sterilized glucose syrup was dissolved in the phosphate solution and this then added to the leucocytes and well shaken. The quantity of glucose-phosphate solution which was added to the leucocytes depended to a certain extent upon the quantity of leucocytes at hand, although no attempt was made to count the cells. The mixture was usually made up to a volume nearly one-half of that of the total exudate. As the exudate contains approximately 10 per cent leucocytes, the glucose was acted upon by a 20 per cent leucocyte suspension. The mixture was kept for thirty-six hours at 37°C. The flasks were stoppered with cotton and well covered and sealed with tin foil to prevent evaporation.

Methods of Analysis Immediately after mixing the leucocytes with glucose and after thirty-six hours samples were withdrawn for analysis. The leucocytes were allowed to settle and the clear supernatant liquid only used for the sugar determinations. The liquid was freed of protein by boiling and acetic acid. The reduced copper was estimated by the Volhard method. The details of the sugar determination are identical with those already described in a previous communication.

Carbon Dioxide A measured volume of the leucocyte mixture was used for this determination according to the method of Fresenius and Classen. The liquid was freed as far as possible from protein by heat while still alkaline. Phosphoric acid was used to acidify the mixture.

Lactic Acid The filtered residue from the carbon dioxide determination was evaporated nearly to dryness. Anhydrous sodium sulphate was then added and all carefully ground in a mortar to an impalpable dry powder. This was then extracted with anhydrous ether, until the extract gave no further test for lactic acid. All extracts were combined and freed of ether. The residue was taken up in a little water and boiled with zinc carbonate. The filtered aqueous solution was evaporated to dryness and the total weight obtained. This residue was usually more or less colored. It was redissolved in water and clarified by boiling with animal charcoal. After evaporating to a small volume zinc lactate soon crystallized. The crystals were used for further analysis and identification.

Volatile Acid The uncoagulated leucocyte mixture was distilled with steam into $\frac{N}{10}$ barium hydrate. Glacial phosphoric acid was added through a separatory funnel in such a manner as

to prevent any loss of CO_2 and volatile acids The adapter from the condenser dipped into the barium hydrate which was contained in a tall stoppered cylinder, connected with a series of wash bottle likewise containing barium hydrate and finally a soda lime tube to guard against absorption of carbon-dioxide through accidental back pressure

A. Experiments showing the relation between rate of glycolysis and concentration of sugar solution

EXPERIMENT I 600 cc exudate was obtained from two dogs Four flasks with glucose at different concentrations were prepared

	CUBIC CENTI METERS USED	CUBIC CENTI METERS NH_4CNS	NH_4CNS PER CUBIC CENTIMETER	PER CENT OF GLUCOSE	LOSS PER 100 CUBIC CENTI METERS	PERCENTAGE LOSS
a At beginning of experiment	0 5	19 0	38 0	13 6		
After thirty-six hours	0 5	19 0	38 0	13 6	0	0
b At beginning of experiment	1 0	30 0	30 0	10 74		
After thirty-six hours	1 0	30 0	30 0	10 74	0	0
c At beginning of experiment	2 0	45 0	22 5	8 05		
After thirty-six hours	2 0	39 5	19 7	7 05	1 00	12 4
After hydrolysis	2 0	39 5	19 7	7 05	0	0
d At beginning of experiment	2 0	36 6	18 0	6 4		
After thirty-six hours	2 0	33 0	16 5	5 9	0 5	7 8
After hydrolysis	2 0	33 0	16 5	0	0	0

EXPERIMENT II 500 cc exudate Four flasks at two different concentrations The remainder 100 cc leucocyte suspension used as control

	CUBIC CENTI METERS USED	CUBIC CENTI METERS NH_4CNS	NH_4CNS PER CUBIC CENTIMETER	GLUCOSE PER 100 CUBIC CENTIMETERS	LOSS	PERCENTAGE LOSS
I At beginning of experiment	1 0	24 4	24 4	8 72		
After thirty-six hours	1 0	22 5	22 5	8 07	0 65	7 5
II At beginning of experiment	1 0	23 9	23 9	8 55		
After thirty-six hours	1 0	22 0	22 0	7 87	0 68	7 9
III At beginning of experiment	1 0	18 5	18 5	6 62		
After thirty-six hours	1 0	16 8	16 8	5 91	0 71	10 7
IV At beginning of experiment	1 0	18 3	18 3	6 55		
After thirty-six hours	1 0	16 5	16 5	5 90	0 65	10 0

B *Experiments aimed to test the formation of volatile acids during the process of glycolysis*

EXPERIMENT IA 100 cc control leucocytes (from A experiment II) acidified and distilled with steam into $\frac{N}{10}$ Ba(OH)₂. The Ba(OH)₂ was filtered from the carbonate and titrated, phenolphthalein used as indicator 20 cc $\frac{N}{10}$ Ba(OH)₂ required 17.6 cc $\frac{N}{10}$ HCl = 2.4 cc

EXPERIMENT IB 99 cc of the leucocyte-glucose mixture was treated in identically the same manner. The 99 cc consisted of—

	SUGAR BEFORE	SUGAR AFTER	LOSS
	grams	grams	grams
I 30 cc	2.61	2.42	0.19
II 27 cc	2.30	2.12	0.18
III 25 cc	1.65	1.47	0.18
IV 17 cc	1.13	1.00	0.13
	7.69	7.01	0.68

37.3 cc $\frac{N}{10}$ Ba(OH)₂ after distillation and filtering from the carbonate required 34.7 cc $\frac{N}{10}$ acid = 2.6 cc $\frac{N}{10}$ neutralized

C *Experiments showing the development of lactic acid in course of glycolysis*

EXPERIMENT IA The residue from B Experiment Ia was extracted in a Schwartz extractor with ether for lactic acid. There was only the merest trace of residue obtained from the ether extract, which was neutral to litmus.

EXPERIMENT IB The residue from B Experiment Ib was extracted for lactic acid. Yield of crude zinc lactate dried at 100° = 0.3184 gram. 0.0961 gram recrystallized salt, air dried after drying

to constant weight lost 0.0106 gram H₂O = 12.3 per cent H₂O

Calculated for two molecules H₂O = 12.5 per cent

0.1362 gram recrystallized salt on ignition yielded

0.0458 ZnO = 33.62 per cent

Calculated = 33.4 per cent

EXPERIMENT II Contents of flasks I and II (A Experiment II) were coagulated and extracted for lactic acid. No lactic acid was obtained from II. The yield from I = 0.305 gram crude zinc lactate

0.2616 gram recrystallized and air dried salt on heating

to constant weight at 100° lost 0.032 gram = 12.35 per cent H₂O

Calculated for two molecules H₂O = 12.5 per cent

0.1135 recrystallized and dried at 110° after ignition

gave 0.0383 ZnO = 33.7 per cent ZnO

Calculated = 33.4 per cent

EXPERIMENT IIIA Contents of flask I Experiment E (170 cc) was coagulated and filtered, the filtrate neutralized and evaporated nearly to dryness. The syrup was acidified with a small quantity of glacial phosphoric acid and

well ground with highest purity anhydrous sodium sulphate This powder was then repeatedly extracted with hot ether The ether extracts were combined and treated as previously mentioned to obtain zinc lactate Yield of crude zinc lactate dried = 0.4798 gram

0.2868 gram recrystallized at 100° dried salt dissolved

in 2.84 cc of water gave a rotation in the polariscope of

— 0.22°

0.3274 gram of the recrystallized salt after drying at

110° weighed 0.2868 gram

Loss = 0.0406 gram

= 12.31 per cent H₂O

Calculated for two molecules H₂O

= 12.5 per cent

0.0999 gram of dried zinc salt gave 0.0337 gram ZnO

= 33.8 per cent ZnO

Calculated

= 33.4 per cent

EXPERIMENT IIIb The controls, flasks II and III Experiment E (170 cc) were subjected separately to the identical treatment as flask I No zinc lactate was obtained

D Experiments showing effect of distilled water and dilution, 700 cc exudate

I Three flasks with 1.86 grams glucose, mixed with leucocytes and phosphate solution

II Two flasks with 3.75 grams glucose with leucocytes and distilled water

III 50 cc leucocytes and phosphate solution set aside for control

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NHCNS	NHCNS PER CUBIC CENTIMETER	GLUCOSE PER 100 CUBIC CENTIMETERS	LOSS	PERCENTAGE LOSS
I At beginning of experiment	4.0	35.0	8.75	3.12		
After thirty-six hours	4.0	32.4	8.1	2.90	0.22	7.8
At beginning of experiment	4.0	32.8	8.2	2.94		
After thirty-six hours	4.0	30.0	7.5	2.68	0.26	8.8
At beginning of experiment	4.0	32.6	8.15	2.92		
After thirty-six hours	4.0	29.6	7.40	2.65	0.27	9.2
II At beginning of experiment	2.0	39.3	19.65	7.04		
After thirty-six hours	2.0	39.4	19.70	7.05	0	0
At beginning of experiment	2.0	35.4	17.7	6.32		
After thirty-six hours	2.0	35.6	17.8	6.33	0	0
III 25 cc of leucocytes and phosphate solution gave no appreciable reduction of Fehling's solution There was likewise no reduction after two hours hydrolysis with 2 per cent HCl						

E. *Effect of adding toluol, control of action of phosphate solution on glucose,
800 cc exudate*

- I One flask of 200 cc glucose, leucocytes and phosphate solution
 II One flask of 200 cc as I, with addition of toluol
 III Glucose and phosphate solution

	CUBIC CENTI- METERS USED	CUBIC CENTI- METERS NH ₄ CNS	NH ₄ CNS PER CUBIC CENTIMETER	GLUCOSE PER 100 CUBIC CENTIMETERS	LOSS	PERCENTAGE LOSS
I At beginning of experiment	2 0	34 0	17 0	6 05		
After thirty-six hours	2 0	31 5	15 75	5 60	0 45	7 4
II At beginning of experiment	2 0	34 3	17 15	6 1		
After thirty-six hours	2 0	34 3	17 15	6 1	0	0
III At beginning of experiment	2 0	35 2	17 6	6 3		
After thirty-six hours	2 0	35 2	17 6	6 3	0	0

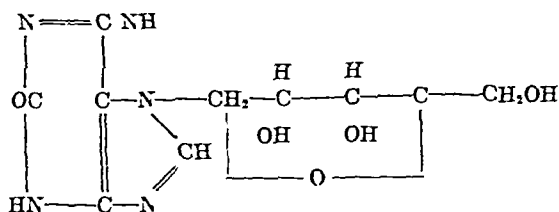
ON THE ACTION OF TISSUE EXTRACTS CONTAINING NUCLEOSIDASE ON α AND β METHYLPENTOSIDES

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Through the work of Levene and Jacobs it was established that the purine bases enter the molecule of the plant nucleic acid and of some animal nucleic acids in the form of a *d*-ribose. The structure of these may be represented by that of guanosine



Regarding two points of their structure there exists at present no definite information. The first concerns the place of the union between the two molecules. The formula given here assumes a union in position 7 of the purine base, but the experimental evidence admits with the same degree of probability also the position 8. The second pertains to the two possible stereoisomeric forms of the pentosides. The assumption of the lactonic structure of glycosides admits of the existence of two isomeric forms of each glycoside conditioned by the asymmetric nature of the end-carbon. Hence theoretically there are possible α and β forms of the nucleosides in the same manner as there exist α and β forms of any other pentoside, and it therefore remains to be established whether the natural nucleosides belong to the α or the β series.

Three methods are available for the solution of the last problem. The first was introduced by Fischer¹ and is based on the specific

¹ *Zeitschr f physiol Chem*, xxvi, p 61, 1898

power of certain enzymes to cause the cleavage of glycosides of one order leaving intact the other stereoisomeric form. Thus emulsin is capable of hydrolyzing β glycosides but not the α forms. On the contrary maltose has no capacity for disrupting the β forms, but possesses one for the α forms. This method was not available in the present investigation, for the reason that neither emulsin nor maltose, in the form as we were able to procure them, had the capacity for cleaving the nucleosides.

The second method was introduced by Armstrong² and is based on the observation of the mutarotation of the sugar liberated from the glycoside. If the mutarotation is analogous to the rotation which characterizes the transformation of the α -isomer into the stable form, the glycoside is regarded as the α -glycoside and *vice versa*. This method is available when there exists an enzyme capable of hydrolyzing the glycoside with a sufficiently high degree of intensity, and besides, when the transformation of the isomeric sugars into their stable forms proceeds at a low rate of velocity. Unfortunately the sugar liberated from the nucleosides possesses a low rotation and is very rapidly transformed into the stable form, so that it shows only a low degree of mutarotation, which becomes evident only under very definite conditions. On the other hand, the cleavage of the nucleosides by the nucleases proceeds very slowly, so that in a moderately short interval only little ribose is liberated. Another difficulty was encountered in the fact that the hypoxanthine formed in course of the cleavage (inosin was used in the experiments) combined with some of the unchanged nucleoside, giving rise to a precipitate of carnin. All these difficulties made the method of Armstrong of little value for the purposes of the present investigation.

The third method was introduced by Hudson,³ and is based on certain numerical values of the rotations of sugars and their glycosides. This method permits of establishing the nature of a glycoside when only one form is known, but when simultaneously there exists information regarding the specific rotation of at least one form of the sugar. According to Hudson the difference in the molecular specific rotation of two sugars has a constant value of

² *Journ Chem Soc*, LVIII, p 1305, 1903

³ *Journ Amer Chem Soc*, LXXI, p 66, 1909

16200 Hence the knowledge of the value of the specific rotation of one form permits of obtaining the value of the other form. Further, the sum of the molecular specific rotations of the two forms remains constant for every sugar and all its glycosides. Thus, if one possesses the knowledge of the sum of the specific rotations of the two forms of a sugar, he is also in possession of the information regarding the sum of the specific rotations of the glycosides. Hence it is possible to calculate by a simple arithmetical process the specific rotation for the second isomeric glycoside when that of the first is known.

Thus accepting the difference in specific molecular rotation expressed by the formula $-\alpha + \beta = \frac{1620}{100} = 108$, and accepting for one isomer $[\alpha]_D = -14.65$, the rotation of the other will equal $-\beta = -\alpha - 108 = -122.65^\circ$. When the rotations of the two forms are given Hudson suggested the following rule for naming the α and β forms: "The names should be selected that for all sugars which are genetically related to *D*-glucose the subtraction of the rotation of the β -form from the α -form gives a positive difference and for all sugars which are genetically related to *L*-glucose an equal negative difference." According to this rule the unknown form of *D*-ribose is to be named the β -form.

The information obtained in this manner furnishes also the value for the sum of the rotations of the two isomers of *D*-ribose, -137.30° , which is also the sum of the rotations of all glycosides of the same sugars. Applying this rule for inosin, of which the known form has the rotation of -49.2° , one is led to the conclusion that the other form has the rotation of -87.80° , and is therefore β -*D*-ribose.

Thus this process of reasoning leads to the conclusion that the natural nucleosides belong to the α series of glycosides. This view may be correct, but in the absence of all other evidence one would hesitate to declare this deduction perfectly conclusive. Hence it seemed desirable to search for additional data that would give more force to the above conclusion or would compel its rejection.

With this aim in view it was attempted to obtain more information regarding the action of the nucleosidases present in the animal tissues. It has been mentioned already that glycosidases of plant origin possess a selective hydrolytic aptitude for only one form of

glucosides and Fischer and Nobel⁴ have demonstrated that the glycosidases of the animal tissues were capable of hydrolyzing only one form of the glucosides, namely the β -form

Hence the α and β forms of methylxylose and methylarabinose were prepared and added to a solution containing the active nucleosidases. Methylribose could not be obtained in crystalline form, and therefore the α and the β forms could not be separated one from another. The efficiency of the enzyme was always tested on nucleosides. To our surprise all tissue extracts failed to act on any one of the pentosides, the ribosides included. The action of the enzyme was tested by the optical method and by the reducing power of the solution for Fehling's solution.

Thus the present experiments failed to contribute to the knowledge of the structure of the nucleosides, but have furnished new information regarding the nature of nucleosidases showing that they possessed a greater degree of specificity than is known to be the property of many glycosidases.

EXPERIMENTAL PART

Organ plasma was prepared in the manner described in a previous communication of Levene and Medigreceanu.⁵ All other details of the experiments were the same as there described.

α -METHYLARABINOSIDE EXPERIMENTS

In neutral phosphate solution (1 per cent)

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA

I, 16, '11	Enzyme solution, 1 cc α -Methylarabinoside solution, 3 cc			
Control	Enzyme solution, 1 cc Phosphate solution, 3 cc			
Experiment	10 min +4 87	24 hrs +4 86	48 hrs +4 86	96 hrs +4 86
Control	0 00	0 00	0 00	0 00

⁴ *Sitzungsberichte Berliner Akad*, v, p 73, 1896

⁵ *This Journal*, 18, p 65, 1911

EXPERIMENT WITH PANCREAS PLASMA

I, 16, '11	Enzyme solution, 1 cc α -Methylarabinoside solution, 3 cc		
Control	Enzyme solution, 1 cc Phosphate solution, 3 cc		
	10 min	24 hrs	96 hrs
Experiment	+5 00	+4 97	+4 96
Control	+0 06	+0 06	+0 06

 α -METHYLXYLOSIDE EXPERIMENTS*In neutral phosphate solution*

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA

I, 16, '11	Enzyme solution, 1 cc α -Methylxyloside solution, 3 cc		
Control	Enzyme and phosphate solution, see α -methylarabinoside experiment		
	10 min	24 hrs	96 hrs
	+2 90	+2 90	+2 89

EXPERIMENT WITH PANCREAS PLASMA

I, 16, '11	Enzyme solution, 1 cc α -Methylxyloside solution, 3 cc		
Control	Enzyme and phosphate solution, see α -methylarabinoside experiment		
	10 min	24 hrs	96 hrs
	+3 00	+2 98	+2 98

 α -METHYLGLUCOSIDE EXPERIMENTS*In neutral phosphate solution (1 per cent)*

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA

II, 9, '11	Enzyme solution, 1 cc Glucoside, 5 per cent, 3 cc		
Control	Enzyme and phosphate solution see maltose experiment		
	10 min	18 hrs	120 hrs
	+3 08	+3 06	+3 05

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EXPERIMENT WITH PANCREAS PLASMA

II, 9, '11	Enzyme solution, 1 cc Glucoside, 5 per cent, 3 cc		
Control	Enzyme and phosphate solution see maltose experiment		
	10 min	18 hrs	120 hrs
	+3 15	+3 16	+3 16

EXPERIMENT WITH KIDNEY PLASMA

II, 9, '11	Enzyme solution, 0 5 cc Glucoside, 5 per cent, 3 0 cc Phosphate solution, 0 5 cc		
Control	Enzyme and phosphate solution, see maltose experiment		
	10 min	18 hrs	120 hrs
	+3 00	+2 98	+3 02

AMYGDALIN EXPERIMENTS

In neutral phosphate solution (1 per cent)

EXPERIMENTS WITH EXTRACT OF INTESTINAL MUCOSA

Experiment I	II, 17, '11	Enzyme solution, 1 cc Amygdalin, 7 per cent, 3 cc			
Control (1)		Enzyme solution, 1 cc Phosphate solution, 3 cc			
Control (2)		Amygdalin solution, 7 per cent			
		10 min	24 hrs	96 hrs	144 hrs
Experiment		-1 10	-1 24	-1 29	-1 32
Control (1)		0 00	0 00	0 00	0 00
Control (2)		-1 48	-1 70	-1 90	-2 00
Experiment II	II, 20, '11	Enzyme solution, 1 cc Amygdalin, 10 per cent, 3 cc			
Control (1)		Enzyme and phosphate solution see preceding experiment			

Control (2) Amygdalin solution, see preceding experiment

10 min	24 hrs	96 hrs	300 hrs
-1 47	-1 62	-1 65	-2 05

Reduced Fehling's solution

EXPERIMENTS WITH PANCREAS PLASMA

Experiment I II, 17, '11 Enzyme solution, 1 cc
Amygdalin, 7 per cent, 3 ccControl (1) Enzyme solution, 1 cc
Phosphate solution, 3 ccControl (2) Amygdalin solution, see experiment with extract
of intestinal mucosa

	10 min	24 hrs	48 hrs	72 hrs	200 hrs
Experiment	-1 05	-1 10	-1 25	-1 35	-1 43
Control (1)	+0 06	+0 06	+0 06	+0 06	+0 06

Experiment II II, 20, '11 Enzyme solution, 1 cc
Amygdalin, 10 per cent, 3 ccControl (1) Enzyme and phosphate solution,
see preceding experimentControl (2) Amygdalin solution, see experiment with extract
of intestinal mucosa

	10 min	24 hrs	96 hrs	300 hrs
Experiment	-1 45	-1 62	-1 85	-2 12

EXPERIMENT WITH KIDNEY PLASMA

Experiment I II, 17, '11 Enzyme solution, 0 5 cc
Amygdalin, 7 per cent, 3 cc
Phosphate solution, 0 5 ccControl (1) Enzyme solution, 0 5 cc
Phosphate solution, 3 5 ccControl (2) Amygdalin solution, see experiment with extract
of intestinal mucosa

	10 min	24 hrs	72 hrs	200 hrs
Experiment	-0 90	cloudy	-1 14	-1 20
Control (1)	-0 04	cloudy	-0 03	-0 03

Experiment II II, 20, '11 Enzyme solution, 0 05 cc
Amygdalin, 10 per cent, 3 cc
Phosphate solution, 0 5 cc

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Control (1)	Enzyme and phosphate solution, see preceding experiment				
Control (2)	Amygdalin solution see experiment with extract of intestinal mucosa				
	10 min	24 hrs	72 hrs	120 hrs	300 hrs
	-1 30	cloudy	-1 60	-1 80	-2 00
	Did not reduce Fehling's solution				

β -METHYLXYLOSIDE EXPERIMENTS

In neutral phosphate solution (1 per cent)

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA

I, 16, '11	Enzyme solution, 1 cc β -Methylxyloside solution, 3 cc		
Control	Enzyme and phosphate solution see α -methyl arabinoside experiment		
	10 min	24 hrs	96 hrs
	-2 60	-2 60	-2 56

EXPERIMENT WITH PANCREAS PLASMA

I, 16, '11	Enzyme solution, 1 cc β -Methylxyloside solution, 3 cc		
Control	Enzyme and phosphate solution, see α -methyl- arabinoside experiment		
	10 min	24 hrs	96 hrs
	-2 00	-2 00	-1 98

β -METHYLARABINOSIDE EXPERIMENTS

In neutral phosphate solution (1 per cent)

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA

I, 16, '11	Enzyme solution, 1 cc β -Methylarabinoside solution, 3 cc		
Control	Enzyme and phosphate solution, see α -methyl- arabinoside experiment		
	10 min	24 hrs	96 hrs
	+0 72	+0 68	+0 68

EXPERIMENT WITH PANCREAS PLASMA

I, 16, '11	Enzyme solution, 1 cc		
	β -Methylarabinoside solution 3 cc		
Control	Enzyme and phosphate solution see α -methylarabinoside experiment		
	10 min	24 hrs	96 hrs
	+0 68	+0 64	+0 63

METHYLRIBOSIDE EXPERIMENTS

In neutral phosphate solution (1 per cent)

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA

II, 2, '11	Enzyme solution, 1 cc			
	Methylriboside solution, 1 cc			
	Phosphate solution, 3 cc			
Control	Enzyme solution, 1 cc			
	Phosphate solution, 3 cc			
	10 min	24 hrs	48 hrs	96 hrs
Experiment	-0 39	cloudy	-0 40	-0 40
Control	0 00	cloudy	0 00	0 00

EXPERIMENT WITH KIDNEY PLASMA

II, 2, '11	Enzyme solution, 0 5 cc		
	Methylriboside solution, 1 5 cc		
	Phosphate solution, 2 5 cc		
Control	Enzyme solution, 0 5 cc		
	Phosphate solution, 4 0 cc		
	10 min	48 hrs	144 hrs
Experiment	-0 50	-0 54	-0 55
Control	-0 06	-0 06	-0 06

EXPERIMENT WITH HEART MUSCLE PLASMA

II, 2, '11,	Enzyme solution, 0 5 cc		
	Riboside solution, 1 cc		
	Phosphate solution, 2 5 cc		
Control	Enzyme solution, 0 5 cc		
	Phosphate solution, 3 5 cc		
	10 min	24 hrs	144 hrs
Experiment	-0 34	-0 37	-0 38
Control	-0 02	-0 02	-0 02

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At the end of the methylriboside experiments, none of the solutions reduced Fehling's solution. However, after the solution had been boiled with H_2SO_4 the sugar test was positive.

d-RIBOSE MUTAROTATION EXPERIMENT

IV, 26, '11		Ribose solution, 10 per cent, in 2 dm long observation tube							Temperature 0°C
		15 min	18 min	25 min	30 min	40 min	45 min	60 min	
Observer I		-2 73		-2 80		-2 98			
Observer II			-2 72		-2 85		-3 01	-3 05	
		18 hrs	20 hrs	24 hrs					
Observer II		-3 44	-3 44	-3 86	(Room temperature)				

STUDIES ON THE ABSORPTION OF METALLIC SALTS BY FISH IN THEIR NATURAL HABITAT

I ABSORPTION OF COPPER BY *FUNDULUS HETEROCLITUS*

By GEORGE F WHITE AND ADRIAN THOMAS

(From the Woods Hole Laboratories of the U S Bureau of Fisheries)

(Received for publication, March 15, 1912)

While it is well known that various fish may take up certain substances dissolved in their surrounding medium, the rate and amount of absorption has not been established very definitely for the most diverse metallic salts. It is our purpose to carry on a complete and comprehensive study of this problem utilizing a large number of salts. The influence of dilution will be noted as well as varying the anion in the different salts studied. Considerable work has been done on the concentration of metallic salts and the time necessary to cause death in certain fish, but our method will be to expose the fish to the salts under conditions approximating as closely as possible those in nature, and to test for the presence of the added substance in the fish taken from the medium while still living. A few isolated experiments have been performed on this line, but we believe that the method of our work is sufficiently improved over these, that repetition if any, is not only desirable but necessary, since a thoroughly systematic view of the field is lacking.

The work of Sollmann¹ on "The Effect of a Series of Poisons on Adult and Embryonic Funduli" may be referred to for the rate of poisoning and degree of toxicity of various substances, the poisons nicotine and digitaline being found to act most rapidly. Also the study carried on by Loeb² and his co-workers of the inhibition by one salt of the poisonous effect of another on *Fundulus*, both

¹ *Amer Journ of Physiol*, xvi, p 1, 1906

² *Biochem Zeitschr*, xxxi, p 450, 1911, xxxii, p 480, 1911

salts being normally present in sea water, is interesting in connection with our study of *Fundulus* although of not direct bearing on our problem. Further, Sumner³ has carried out some careful and complete investigations of the osmotic relations between *Fundulus* and other fish and their surrounding medium. The relative toxicity of various poisons in different media was studied, and such substances as cupric chloride were found to be less fatal in sea water of high salt content, or fresh water to which sugar had been added, than water of low salt or sugar content. These results seem to conclusively prove that it was the increase of the osmotic pressure in the surrounding medium by the addition of these substances, which prevented the toxic action of the salt.

The fish selected for our experiments was *Fundulus heteroclitus*, for many reasons. It is small and abounds in the shallow waters around Woods Hole, and is a hardy fish, living practically indefinitely in tanks through which sea water is kept running. Of course this latter arrangement would be impossible for experiments of the character described in this paper, but it was found that the following method was successful.

Large glass vessels, of about 10 liters capacity, were filled with sea water containing the desired amount of salt—copper sulphate—and during the whole period while the fundulus were kept in these, a constant stream of air was blown in, furnishing sufficient oxygen for the fish to live for days. The aeration was conveniently accomplished by drawing off the excess air through a tube from a bottle attached to the water outlet of a Richards suction pump, the water being siphoned out of the bottle through another tube. Care was taken not to cause "air-sickness" by the use of too strong a blast. By this process, the only disturbing influence, outside of the added poison, was the accumulation of the excretory products from the fish, which difficulty was obviated or lessened by changing the medium about every twelve hours.

Fifteen to twenty *Fundulus* were placed in each of several vessels, and after being subjected to the action of the poison for the desired length of time, were taken out while still active and prepared for analysis. They were thoroughly washed with fresh water and a stream of water was also passed through the alimentary

³ Biol. Bull., x, p. 298, 1906, Amer. Journ. of Physiol., xix, p. 61, 1907.

tary tract to remove all traces of copper sulphate not actually absorbed in the body of the fish. They were then cut up in small pieces and analyzed for copper as follows

The fish were dried to constant weight at 110° to 120° and about 10 grams of the dried flesh taken for analysis. Each sample was placed in a Kjeldahl flask with 80 to 100 cc conc sulphuric acid and 5 grams of potassium sulphate. A small amount of paraffin was added to prevent excessive frothing. The solutions were digested until they became clear, this process requiring between ten and eighteen hours. The conditions of digestion were therefore similar to the Gunning method for the analysis of proteins for nitrogen, in consequence of the presence of the absorbed copper. Oxidation of the organic matter by treatment with concentrated nitric acid was previously tried, but found unserviceable due to uncontrollable frothing.

The solutions were diluted to 200 cc, a few drops of phenolphthalein solution added, and nearly neutralized with 50 per cent sodium hydroxide solution. The solution, measuring about 250 cc, was then electrolyzed with a current of 0.5 to 0.75 ampere and a potential difference of 2.5 volts. The current was obtained from two storage batteries set up in series. The electrolysis was allowed to proceed for five hours at which time it was complete, trial having shown this to be sufficient. The cathodes were then dried and weighed in the usual manner.

In order to prove that there was no other deposit on the electrode than copper, blank tests were made on normal *Fundulus*, that is *Fundulus* not having been placed in a copper sulphate solution. Several of these tests were made during the course of the experiments, and absolutely no deposit was found on the cathode in any case.

Moisture determinations were made on the *Fundulus* flesh so that the percentage of copper absorbed could be calculated either for the original flesh or the dried material, this latter being the most desirable. The results of six such analyses of normal *Fundulus* are as follows: 77.30, 77.33, 77.49, 76.77, 78.19, 77.12. Average, 77.43 per cent.

The results of the poison experiments are given in Table I, the strength of the solutions being referred to normal, and the amount of copper absorbed being expressed in percentage by weight of metallic copper in the dried flesh. A summary of the results is presented in Table II, percentage of copper being calculated for the undried and the dried flesh.

It may be seen from the data that very appreciable amounts of copper were taken up by the *Fundulus*, even although the most concentrated solutions were practically of low copper content. Scaly fish may then absorb poison to a degree which is of the same

TABLE I
Copper absorbed by Fundulus

NORMALITY	TIME	COPPER IN DRIED FLESH	NORMALITY	TIME	COPPER IN DRIED FLESH
	hours	per cent		hours	per cent
$\frac{1}{250}$	1	0 0210	$\frac{1}{1000}$	4	0 0200
		0 0160			0 0190
		0 0110			0 0083
	2	0 0212		6	0 0086
		0 0100			0 0080
		0 0213			0 0070
$\frac{1}{500}$	3	0 0190	$\frac{1}{2000}$	2	0 0070
					0 0114
					0 0110
	1	0 0100		4	0 0080
		0 0100			0 0170
		0 0060			0 0060
$\frac{1}{1000}$	2	0 0650	$\frac{1}{4000}$	8	0 0070
		0 0128			0 00500
		0 0200			0 00427
	3	0 0360		24	0 00600
		0 0100			0 0070
					0 0060
$\frac{1}{1000}$	1	0 0040	$\frac{1}{8000}$	48	0 00299
		0 0030			0 00310
		0 0060			0 00400
	2	0 0070		24	0 00300
		0 0088			0 00400
		0 0193			0 00400
	3	0 0080		96	0 00400

order of magnitude as oysters, which Bothe⁴ has shown have taken up under natural conditions from 0 017 per cent to 0 050 per cent of their body weight in copper, such flesh containing at the same time more water than the Fundulus. The greatest absorption with the Fundulus takes place in the first part of the period of exposure to the poison, and there is a gradual increase with length of time until enough has been accumulated to seriously affect the life of the fish. As much absorption may occur in the dilute solutions as in the concentrated if sufficient time is allowed, thus there is as great a percentage of copper in the flesh after an experiment of four hours duration in $\frac{1}{1000}$ solution as after three hours in a $\frac{1}{250}$

⁴ Amer Food Journ, vi, p 2, 1911

solution, and a larger percentage after four hours in a $\frac{N}{500}$ solution. In the extremely dilute solution of $\frac{N}{8000}$ normality, ninety-six hours is required to produce an accumulation of 0.0040 per cent of copper.

TABLE II
Average results of absorption experiments

NORMALITY	TIME	CU IN DRIED FLESH	CU IN UNDRIED FLESH
	<i>hours</i>	<i>per cent</i>	<i>per cent</i>
$\frac{1}{50}$	1	0.0160	0.00361
	2	0.0156	0.00352
	3	0.0201	0.00454
$\frac{1}{300}$	1	0.0100	0.00226
	3	0.0164	0.00360
	4	0.0230	0.00529
$\frac{1}{1000}$	1	0.0035	0.00079
	2	0.0065	0.00147
	3	0.0103	0.00232
$\frac{1}{2000}$	4	0.0195	0.00440
	2	0.0075	0.00169
	3	0.0120	0.00269
$\frac{1}{4000}$	4	0.0125	0.00280
	24	0.00509	0.00115
	48	0.00650	0.00147
$\frac{1}{8000}$	24	0.00306	0.00069
	48	0.0035	0.00079
	96	0.00400	0.00090

Since fish take up the considerable amount of copper shown by our experiments, it may be asked in what manner this takes place. To answer this tentatively, a brief study of the distribution of the absorbed salt in the fish was made. Since the *Fundulus* is so small that it is difficult to dissect and separate its organs, the larger tautog (*Tautoga onitis*) was selected. This was placed in $\frac{N}{1000}$ copper sulphate solution for two hours under the same conditions as the *Fundulus*, and analysis made for copper in the blood system—heart, gills, blood vessels—the alimentary tract—stomach, intestines, etc.—and the flesh. The results are given in Table III.

The amount of copper taken up by the fish, 0.007 per cent of its total body weight (dry), is practically identical with the result for *Fundulus*, 0.0065 per cent, obtained in the same dilution and for

TABLE III

Distribution of copper absorbed by Tautog in $\frac{N}{1000}$ solution

MATERIAL	PER CENT COPPER IN DRY MATERIAL		
	I	II	AVERAGE
Whole fish	0 008	0 006	0 007
Blood system	0 010	0 016	0 013
Alimentary tract	0 003	0 001	0 002
Flesh	0 009	0 011	0 010
Residue	0 005		0 005

the same time period. Therefore we may assume that the two species do not act materially differently towards the poison. The results are quite positive in their character. The largest percentage of copper was found in the blood system and it is therefore reasonable to conclude that it is *through the gills*, where the surrounding medium comes in most intimate contact with the blood, that the absorption is the greatest. A final and absolute statement of this we do not put forward, since this one experiment is not sufficient to firmly establish any theory. But nevertheless it is very suggestive. Taken in connection with the work of Scott and White⁵ on the permeability to salts of the gill membranes of a fish, it receives some support.

It is interesting to note that we found visible evidence of the copper in the fish, the tautog especially showing the green color caused by the reaction between the copper sulphate and the protein substance.

Microscopic sections⁶ of the whole Fundulus (cross-section), and even of the brain and spinal cord, treated with potassium ferrocyanide, were colored markedly brown by the formation of copper ferrocyanide, whereas normal Fundulus evidenced no such change.

Work on the problem of absorption of salts will be continued.

⁵ *Science*, **xxii**, p 768, 1910.

⁶ The tissues, preserved in alcohol, were embedded in paraffin, cut with the microtome, the paraffin dissolved in turpentine which latter was removed by alcohol. After washing out the alcohol with water to prevent precipitation of $K_4Fe(CN)_6$, the sections were washed in $K_4Fe(CN)_6$, washed with water and alcohol, and mounted in balsam.

THE DETERMINATION OF ALUMINUM IN FECES

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(Received for publication, March 16, 1912)

On looking about for a satisfactory method for determining aluminum in the feces of experimental subjects who were given either alum or the aluminum-containing residue from baking powder, we encountered a number of difficulties. The determination of aluminum as the hydroxide was impossible on account of the presence of phosphates. The determination of aluminum by precipitation both of iron and aluminum as phosphates and then determining the iron and the phosphorus, determining the aluminum by difference, is too long and besides, the value for aluminum so obtained may include the combined errors of the other two determinations. In attempting to use the Peter's method,¹ in which iron is reduced by ammonium thiosulphate and the aluminum determined as the phosphate, we encountered a number of difficulties, yet this method gives a direct determination of aluminum, so the attempt was made to find out the conditions under which it could be used. Substances present in the feces which affect the determination of aluminum by this method are Organic matter, silica, tin (from canned foods), iron, calcium and phosphates.

Organic matter can be removed by mixing with the feces several cubic centimeters of concentrated sulphuric acid and ashing in a silica dish. All of the aluminum in the ash is not soluble in hydrochloric acid. It is necessary to dissolve out the acid-soluble part and then fuse the insoluble residue with sodium carbonate.² The silica is dehydrated and the solution added to the main portion. Tin can be removed by precipitation as the sulphide in an acid

¹ Circular 26, U S Bureau of Standards

² *Ibid* , p 6

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solution Complete removal of the iron and probably calcium also, is not accomplished in the first precipitation of the aluminum. On redissolving the precipitate in hydrochloric acid and again precipitating the aluminum as the phosphate, only a negligible trace of iron remains in the precipitate. A large excess of ammonium phosphate should be avoided since it is difficult to wash out and the time of heating the precipitate to constant weight is thereby greatly increased.

The latter process necessitates a very high temperature. A small precipitate may be brought to a constant weight by heating over a blast lamp for an hour, but for precipitates of 150 mg or more, a higher temperature or a greatly prolonged heating is necessary. We have found that on heating precipitates in a Méker muffle furnace at a dull white heat for an hour to an hour and a half, precipitates as high as 300 mg can be brought to constant weight. Porcelain crucibles cannot be used. Platinum, while remaining constant in weight, soon crystallizes and is rendered worthless. A glazed silica crucible will remain constant in weight for several determinations, but on prolonged heating will lose weight. A transparent silica crucible will remain constant somewhat longer. The empty crucible should be cleaned and weighed after being used, to make sure that there has been no loss in weight.

The details for carrying out a determination of aluminum in feces are as follows. Five to ten grams of feces are treated with several cubic centimeters of concentrated sulphuric acid and ashed in a silica dish. The soluble aluminum is dissolved out by warming with dilute hydrochloric acid. The residue on the filter paper is washed and then ignited and fused with sodium carbonate in a platinum crucible. The melt is dissolved out with dilute hydrochloric acid, the silica dehydrated, and the whole added to the main portion containing the aluminum. The volume at this point should be about 300 cc, and contain about 25 cc of concentrated hydrochloric acid. Tin is precipitated from the hot solution by hydrogen sulphide and filtered off. Di-ammonium hydrogen phosphate is added to the solution—0.5 gm for each 100 mg of aluminum phosphate present. The solution is heated, and while hot 5 grams of ammonium thiosulphate (in solution) and after several minutes 6 to 8 grams of ammonium acetate (in solution) and 4 cc strong acetic acid are added. Heating is

continued for about half an hour to expell SO_2 , the precipitate allowed to settle, filtered and washed once by decantation The precipitate is redissolved in 2 to 2.5 cc of concentrated hydrochloric acid, the solution diluted to about 300 cc, 0.5 gram of ammonium phosphate added for each 100 mg of aluminum phosphate present and the aluminum again precipitated as described above The precipitate is filtered and washed several times with hot water to remove chlorides and ignited in a transparent silica crucible until constant weight is reached to remove excess of P_2O_5

The precipitate obtained in this manner is easily filtered and washed The free sulphur present serves to make the precipitate more flocculent and more easily filtered By using ammonium salts throughout as precipitating reagents the necessity for very thorough washing of the precipitate is eliminated, since small amounts of such salts remaining in the precipitate are volatilized on ignition Washing the precipitate with ammonium nitrate has been recommended, but we have found such procedure unnecessary The minimum amount of hydrochloric acid necessary to keep the aluminum in solution before precipitation, should be used, thus making it easier to reduce its concentration in the aluminum precipitation The method carried out as above gives good check results.

Using this method with various amounts of aluminum in feces and in solutions containing known amounts of aluminum we obtained results as follows

(1) Solution of pure AlCl_3 (single precipitation)

	FOUND		CALCULATED
	AlPO_4	Equivalent Al_2O_3	Theoretical Al_2O_3
(a)	0.1721	0.0720	0.0720
	0.1723	0.0721	
	0.1718	0.0719	
(b)	0.0106	0.0044	0.0044
	0.0106		

(2) A known volume of AlCl_3 was added to 50 cc of a mixture containing per liter the following salts

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	Grams
Sodium phosphate	25
Potassium chloride	15
Ferric chloride	5
Calcium chloride	11
Magnesium chloride	11

FOUND		CALCULATED
AlPO ₄	Equivalent Al ₂ O ₃	Theoretical Al ₂ O ₃
0 1677	0 0702	0 0707
0 1684	0 0705	
0 1686	0 0706	

(3) Duplicate samples of dried feces of men who were given alum gave the following results

	AlPO ₄	Equiva- lent Al		AlPO ₄	Equiva- lent Al
(a)	{ 0 1799	0 0399	(e)	{ 0 0915	0 0203
	{ 0 1825	0 0405		{ 0 0902	0 0200
(b)	{ 0 1942	0 0431	(f)	{ 0 0333	0 0074
	{ 0 1930	0 0428		{ 0 0348	0 0077
(c)	{ 0 1848	0 0410	(g)	{ 0 2143	0 0476
	{ 0 1846	0 0410		{ 0 2143	0 0476
(d)	{ 0 2141	0 0475			
	{ 0 2120	0 0471			

(4) Determinations of aluminum in feces of different subjects on a constant diet, but who were not given any aluminum salt, gave the following results, as total amounts of aluminum excreted in a period of two weeks

Subject A

	Gram Al
First two weeks	0 040
Second two weeks	0 053
Third two weeks	0 063
Fourth two weeks	0 054
Fifth two weeks	0 060

This is equivalent to an elimination of 3 to 4 mg of Al per day

(5) The necessity for a high temperature, and prolonged heating of the precipitate to get a constant weight is shown by the following results of duplicate determinations

- AlPO_4
gram
- (a) $\left\{ \begin{array}{l} 0.2817 \\ 0.2836 \end{array} \right\}$ Precipitates heated for one hour in gas muffle at a bright red heat
- (b) $\left\{ \begin{array}{l} 0.2764 \\ 0.2768 \end{array} \right\}$ Same precipitates heated at low white heat in Méker furnace for fifteen minutes
- (c) $\left\{ \begin{array}{l} 0.2754 \\ 0.2765 \end{array} \right\}$ Same precipitates further heated at white heat in Méker furnace for fifteen minutes
- (d) $\left\{ \begin{array}{l} 0.2757 \\ 0.2765 \end{array} \right\}$ Same precipitates heated at white heat in Méker furnace for thirty minutes more. The precipitates were ignited in platinum crucibles. No change in weight in the crucibles was noted. A triplicate determination heated in the same way, but in a porcelain crucible gave a value of 0.2840 gram AlPO_4 . The excess weight in this case apparently results from a combination of the P_2O_5 with the glaze of crucible, rendering a correct determination impossible.

Determinations of phosphorus made on 0.0625 gram AlPO_4 gave results as follows

	gram		gram
P (found)	$\left\{ \begin{array}{l} 0.0161 \\ 0.0161 \end{array} \right\}$	P (calculated)	0.0159

We are indebted to Mr F P Veitch for his paper on "Experiments on the Estimate of Iron and Aluminum in Phosphates" which he kindly submitted to us during the progress of our work

RESEARCHES ON PURINES

ON 2,8-DIOXY-6,9-DIMETHYLPURINE AND 2,8-DIOXY-1-METHYLPURINE

SIXTH PAPER ¹

BY CARL O. JOHNS

(From the Sheffield Laboratory of Yale University)

(Received for publication, March 19, 1912)

Although the dioxy-dimethyl-purines are of considerable interest owing to the fact that they are isomeric with theobromine,² 2,6-dioxy-3,7-dimethylpurine (IX), yet very few of the many possible isomers have been described. Of the nine isomerides of 2,8-dioxy-dimethylpurine only one member has been described, namely, 2,8-dioxy-3,7-dimethylpurine (XII). This compound was obtained by Emil Fischer³ who chlorinated 3,7-dimethyluric acid (X) and reduced the resulting chloride (XI) with hydriodic acid.

In a previous contribution⁴ from this laboratory it was shown that orthodiaminopyrimidines, in which a hydrogen atom of an amino group has been substituted by an alkyl group, condense readily with formic acid or urea to form purines. This method has now been applied in the synthesis of 2,8-dioxy-6,9-dimethylpurine (IV).

2-Ethylmercapto-4-methyl-6-chloropyrimidine⁵ (I) was heated in a sealed tube with methylamine and the result was a quantitative yield of 2-ethylmercapto-4-methyl-6-methylaminopyrimidine (II). This, in turn, was converted to 2-oxy-4-methyl-6-methylaminopyrimidine (III), which, when nitrated, gave 2-oxy-4-methyl-

¹ This *Journal*, vi, p 73, 1912

² *Beilstein's Handb*, iii, p 954

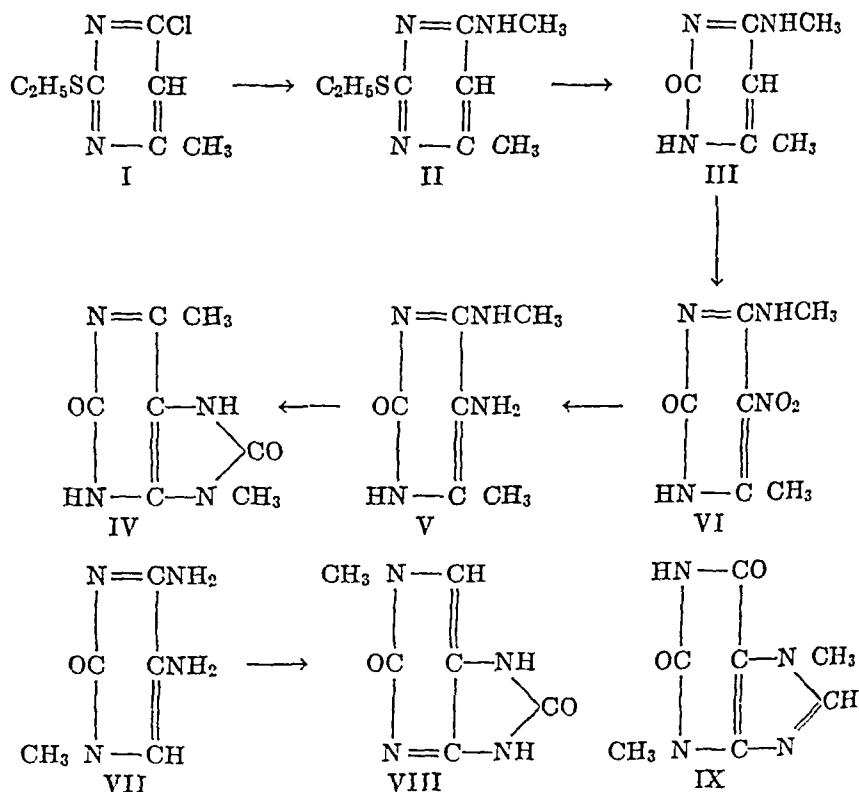
³ *Ber d deutsch chem Gesellsch*, xxviii, p 2487, 1895, xxx, p 1851, 1897, xxxii, p 474, 1899

⁴ Johns *This Journal*, ix, p 161, 1911

⁵ *Amer Chem Journ*, xl, p 351, 1908

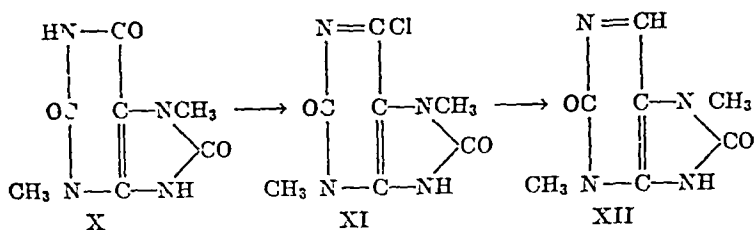
5-nitro-6-methylaminopyrimidine (VI) The yields were satisfactory When the nitro-compound was reduced with freshly precipitated ferrous hydroxide an 83 per cent yield of 2-oxy-4-methyl-5-amino-6-methylaminopyrimidine (V) was obtained By heating with urea, the diamino-compound was easily converted to 2,8-dioxy-6,9-dimethylpurine (IV)

This paper also contains a description of the synthesis of 2,8-dioxy-1-methylpurine (VIII), which compound was prepared by heating urea with 2-oxy-3-methyl-5,6-diaminopyrimidine⁶ (VII) As three of the isomers of 2,8-dioxy-monomethylpurine have been described previously,⁷ the only member of this series which is still unknown is 2,8-dioxy-7-methylpurine These researches will be continued

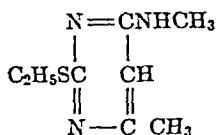


⁶ Johns *This Journal*, vi, p 77, 1912

⁷ Fischer and Ach *Ber d deutsch chem Gesellsch*, xliii, p 2736, 1899, Johns *This Journal*, i, p 63, 1909, *Amer Chem Journ*, xli, p 63, 1909

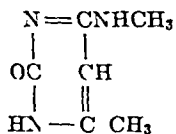


EXPERIMENTAL PART

2-Ethylmercapto-4-methyl-6-methylaminopyrimidine

Fifteen grams of 2-ethylmercapto-4-methyl-6-chloropyrimidine⁸ were mixed with 21 cc of a 33 per cent aqueous solution of methylamine and 30 cc of water and this mixture was heated in a sealed tube at 100°C over night. A heavy transparent oil formed and this solidified to a white crystalline mass on cooling. The reaction product, thus obtained, was easily soluble in cold ether, benzene or alcohol but it was almost insoluble in hot water. It dissolved readily in cold concentrated hydrochloric acid. When crystallized from dilute alcohol it formed beautiful, flat, anhydrous prisms that melted to an oil at 87°C. The yield was quantitative.

	Calculated for $\text{C}_8\text{H}_{11}\text{N}_3\text{S}$	Found
N	22.95	23.03

2-Oxy-4-methyl-6-methylaminopyrimidine

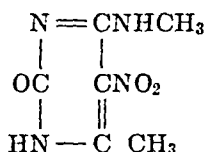
Twenty grams of 2-ethylmercapto-4-methyl-6-methylaminopyrimidine were dissolved in 200 cc of concentrated hydrochloric

⁸ Johns *Loc cit*

acid and the solution was evaporated to dryness on the steam-bath. The residue was then found free from sulphur. It was dissolved in hot water and the solution was made slightly alkaline with ammonia whereupon crystals formed rapidly. These were easily soluble in cold acetic acid and slightly soluble in hot alcohol. They were moderately soluble in hot water from which solvent they separated in compact, biconcave, anhydrous blocks. These turned brown at about 290°C and decomposed slowly above that temperature. The yield was 83 per cent of the calculated.

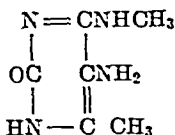
	Calculated for $C_6H_8ON_4$	Found
N	30.21	30.00

2-Oxy-4-methyl-5-nitro-6-methylaminopyrimidine



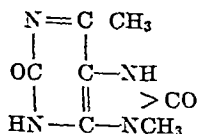
Twelve grams of 2-oxy-4-methyl-6-methylaminopyrimidine were dissolved in 30 cc of concentrated sulphuric acid. While this solution was kept cool, 4.8 cc of nitric acid (density 1.5) were added gradually. After five minutes the resulting solution was poured on crushed ice and the acids were neutralized with ammonia. A crystalline precipitate separated at once. The yield was quantitative. The nitro-compound was practically insoluble in hot alcohol and but slightly soluble in hot water. It dissolved moderately in glacial acetic acid. By cooling this solution slowly small, stout prisms were obtained, while by cooling rapidly slender, pointed crystals were formed. The crystals turned dark at about 250°C and decomposed slowly when heated above that temperature.

	Calculated for $C_8H_{10}O_2N_4$	Found
N	30.43	30.12

2-Oxy-4-methyl-5-amino-6-methylaminopyrimidine

Fourteen grams of 2-oxy-4-methyl-5-nitro-6-methylaminopyrimidine were dissolved in a mixture of 200 cc of concentrated ammonia and an equal volume of water. A concentrated aqueous solution containing 174 grams of crystallized ferrous sulphate was added. Ferric oxide was precipitated rapidly, the reaction being exothermic. The sulphate was precipitated by the addition of a solution of 203 grams of crystallized barium hydroxide and the excess of baryta was removed by means of ammonium carbonate. After shaking a few times, the mixture was set aside over night, after which it was heated to about 80°C and filtered by suction, the precipitate being washed with hot water. On evaporating the filtrate to dryness a crystalline crust was left. This was dissolved in hot water and the color was removed with blood coal and, on cooling, a bulky mass of colorless, anhydrous needles was obtained. These charred rapidly above 270°C. They were soluble in less than ten parts of boiling water, easily soluble in cold glacial acetic acid and slightly soluble in hot alcohol. The yield was 83 per cent of the calculated.

N	Calculated for	Found
	$\text{C}_6\text{H}_{10}\text{ON}_4$	
	36.36	36.53

2,8-Dioxy-6,9-dimethylpurine

Two grams of urea and an equal weight of 2-oxy-4-methyl-5-amino-6-methylaminopyrimidine were ground together and the mixture was heated in an oil-bath at 180 to 190°C for an hour. The mass first melted but soon solidified. The reaction-product

was dissolved in dilute ammonia and decolorized with blood coal. After boiling off most of the ammonia, the solution was acidified with acetic acid. Compact prisms were formed by cooling the solution slowly, but by cooling rapidly a bulky mass of needles was obtained. The yield was quantitative. The purine dissolved in about sixty parts of boiling water but was difficultly soluble in cold water. It was almost insoluble in boiling alcohol. It dissolved readily in dilute alkalis. It possessed the usual stability of dioxypurines and did not melt at 320°C. The crystals contained 2 molecules of water.

1. 193 grams of substance lost 0.1965 gram at 135°C

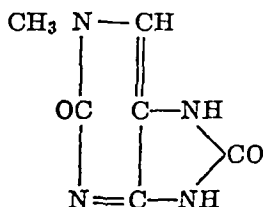
	Calculated for $C_7H_4O_2N_4 \cdot 2H_2O$	Found
H ₂ O	16.66	16.47

0.2204 gram of anhydrous substance gave 0.0891 gram of H₂O and 0.3772 gram of CO₂

	Calculated for $C_7H_4O_2N_4$	Found
C	46.66	46.66
H	4.44	4.49
N	31.11	31.14

This purine did not form a picrate. It could be crystallized from an aqueous solution of picric acid and the crystals formed were those of the free base. When crystallized from 20 per cent hydrochloric acid it gave a hydrochloride that was easily soluble in hydrochloric acid and that hydrolyzed readily in water. When warmed with 30 per cent nitric acid it was oxidized and on evaporating the solution a red residue was obtained. This turned a brilliant purple when moistened with ammonia.

2,8-Dioxy-1-methylpurine



A mixture of pulverized urea and an equal weight of 2-oxy-3-methyl-5,6-diaminopyrimidine⁹ was heated in an oil-bath at 170 to 180°C for an hour. The reaction product was dissolved in hot dilute ammonia and the solution was decolorized with blood coal. On acidifying with acetic acid the purine crystallized from the hot solution in the form of small, anhydrous plates. These did not melt at 320°C. The yield was almost quantitative. The purine was difficultly soluble in cold water and almost insoluble in boiling alcohol. One part of the purine dissolved in about 200 parts of boiling water. It was soluble in concentrated acids but the salts dissociated in water.

0.1782 gram of substance gave 0.2852 gram of CO₂ and 0.0610 gram of H₂O

	Calculated for C ₆ H ₆ O·N ₄	Found
C	43.37	43.64
H	3.61	3.80
	I	II
N	33.73	33.77 33.60

⁹ Johns *Loc cit*

THE CHEMICAL ANALYSIS OF THE ASH OF SMOOTH MUSCLE

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The salts dissolved in the tissue fluids of animals and plants play an important physiological rôle, and a large amount of work has been done in the last twenty years with the object of discovering the nature of these salts and the manner of their combination in different tissues. The usual method of dealing with these problems has been to make ash analyses, the blood, lymph, and striated muscle of many animals have been analyzed in this manner.

Very few analyses, however, have been made of the ash of smooth muscle. Kühne states that it is richer in sodium than in potassium,¹ and this statement has been quoted in later text-books and in scientific articles. But Kühne gives only the bare statement quoted above with no reference and no account of the work on which it is based. Neumeister, on the other hand, says, "Die glatten Muskeln zeigen in ihrem chemischen Verhalten von den quergestreiften kaum Abweichungen," and the context makes it appear that this statement refers, among other things, to ash analysis.² But here also there is no reference and no further account of work on which the statement might be based. Halliburton makes a similar though somewhat vaguer unsupported statement.³ Finally, Macallum in a recently published general article says, "Analysen des Natriums und Kaliums im glatten Muskel zeigen,

¹ Kühne *Lehrbuch der physiologischen Chemie*, Leipzig, p. 333, 1868.

² Neumeister *Lehrbuch der physiologischen Chemie*, 2 Aufl., Jena, p. 442, 1897.

³ Halliburton *Text-book of Chemical Physiology and Pathology*, London, p. 398, 1891.

dass letzteres reichlicher als ersteres vorhanden ist, obgleich das Ueberwiegen nicht so gross ist, wie in der quergestreiften Faser" In a footnote he says that this statement is based on unpublished data of his own ⁴

We are familiar with two accounts of more or less complete analyses of the ash of smooth muscle. The first of these is by Saiki⁵ who worked on the stomach and bladder muscle of the pig and found the ash of these tissues widely different from that of the striated muscle of the same animal. Saiki finds considerably more sodium than potassium in the pig's smooth muscle, and much less phosphorus and sulphur and more chlorine than earlier investigators have found in the striated muscle of the same animal. According to Saiki, therefore, the ash of smooth muscle is a good deal more like that of the blood plasma than is that of striated muscle.

But Costantino⁶ has recently analyzed the smooth muscle of various animals for sodium, potassium, and chlorine, and finds in general that the potassium is much higher than the sodium, though the difference is usually less marked than it is in striated muscle. Costantino has analyzed the retractor penis of the pig for chlorine and gets a figure somewhat lower than Saiki's. Saiki's figures are compared with those of Costantino in Table I, and Katz's⁷ figures for the striated muscle of the pig and ox are given in the same table.

There are various possible explanations for the differences between Saiki's results, on the one hand, and those of Costantino and Macallum on the other. According to the figures given by Katz the pig is a rather exceptional animal in regard to the sodium and potassium content of its striated muscle. Katz analyzed the ash of striated muscle in the human being, pig, steer, calf, stag, rabbit, dog, cat, chicken, frog, haddock, eel and pike. In the pig there is less than twice as much potassium as sodium, while in the other animals there is from three to fourteen times as much. It may be that pig's smooth muscle is even more different from that of other animals in this respect than is its striated muscle. It

⁴ Macallum *Ergeb d Physiol*, p 642, 1911

⁵ Saiki *This Journal*, iv, p 483, 1903

⁶ Costantino *Biochem Zeitschr*, xxvii, p 52, 1911

⁷ Katz *Arch f d ges Physiol*, lxii, p 1, 1896

TABLE I
Constituents of smooth and striated muscle given as percentages of the fresh tissue

	K	Na	Po	Cn	Mg	P	Cl	S	SOLIDS	WATER
<i>Smooth muscle</i>										
Sauki's averages for the stomach and bladder of the pig	0 037	0 243	0 008	0 032	0 001	0 081	0 171	0 087	20 4	79 6
Costantino { Ox stomach	0 305	0 089					0 104			
{ Ox retractor penis							0 128			
Pig retractor penis	0 207	0 109					0 130			
<i>Striated muscle</i>										
Katz { Pig	0 254	0 150	0 000	0 008	0 028	0 213	0 018	0 201	27 11	72 98
{ Ox	0 300	0 095	0 021	0 002	0 024	0 170	0 057	0 187	21 20	75 80

seems strange, however, that the smooth and striated muscle of the pig should be so much more markedly different in sodium and potassium content than are the two kinds of muscle in other animals, and that the magnesium, phosphorus, and sulphur content of the pig's smooth muscle should be so low. Katz did not find the pig's striated muscle exceptional in respect to its content of these three elements. If one studies the sodium and potassium figures which Saiki gives for his individual analyses one finds a rather wide variation, the sodium varies from 0.2 per cent to a little over 0.3 per cent and the potassium all the way from 0.039 per cent to 0.081 per cent. In the text of Saiki's article (p. 492) is the statement, "A comparison of the composition of fresh pig's muscle of different types with blood serum (resembling lymph) of the same species, indicates that the assumption of an admixture of lymph may explain the higher content of sodium and chlorine and the lower percentages of potassium, magnesium and phosphorus." This statement taken in connection with the rather wide variation in Saiki's figures for sodium and potassium brings up the question how much of the tissue analyzed by him was really smooth muscle.

Another fact that must be taken into account is that Saiki extracted his tissue with ether before ashing it, while Costantino did not. It is quite possible that preliminary extraction with ether would lower the phosphorus content in the ash of a tissue.⁸

We have made analyses of the ash of frog's stomach muscle. We have chosen the frog as the object in our investigation, partly because the tissues of this animal have become the standard in all sorts of physiological experimentation, and partly because it is easy to obtain from the frog's stomach a tissue which is undoubtedly 90 per cent irritable smooth muscle at the time the chemical investigation is begun. We have used the large American bullfrog (*Rana Catesbeiana*), the stomach of a single individual of this species sometimes yields more than 2 grams of smooth muscle.

Our muscle was prepared as follows. From six to eighteen frogs were killed and the stomachs were dissected out. The stomachs were never left in the dead frogs for more than three hours, and usually for a much shorter period. The muscle was always still irritable at the time the chemical examination

⁸ See Katz *Loc cit* pp. 9 and 10.

was begun. The stomachs were laid on a glass plate and the muscular coats were cut open along the line of the lesser curvature, while the mucous membrane was left as a still unopened tube. The mucous tube was then torn loose from the muscle, and in this way contamination of the muscle with stomach contents was avoided. After the separation of the mucous and muscular layers of the stomach, the small amount of submucous connective tissue which usually adheres to the inner surface of the muscle was stripped off. The sheets of muscle so obtained were pressed several times against hardened filter paper, weighed, and then either fused, or dried and incinerated, according to the nature of the analysis that was to be carried out.

If such sheets of muscle as we used for analysis are fixed and examined histologically, it will be found that they contain from 90 to 95 per cent of smooth muscle and from 5 to 10 per cent of serous connective tissue. The same relations are found if one examines slices of the fresh tissue, and it is probable, therefore, that the "smooth muscle" which we used for analysis contained less connective tissue than the "striated muscle" used by Katz and the other investigators of the inorganic constituents of striated muscle.

Saiki suggests (p. 492) that smooth muscle may contain larger lymph spaces than striated muscle. We have made a careful investigation of this question. Samples of muscle were fixed in various ways and embedded in paraffin. From these, thin transverse sections were cut and stained by methods which are supposed to be specific for smooth muscle. From other samples cross sections were sliced off free hand with a sharp razor and examined microscopically in Ringer's solution. Such sections were often stimulated by an electric current beneath the microscope, and the muscle in them usually contracted, showing that they were still alive.

In both fixed and living preparations it was found that the muscle fibers occupied from 80 to 90 per cent of the total volume of the preparation, and the interstitial spaces, from 10 to 20 per cent. The lymph spaces between the fibers are therefore smaller in the smooth muscle of the frog's stomach than they usually are in striated muscle. We are prepared to assert that at least 75 per cent of the total volume of our tissue was occupied by the muscle fibers, and we should judge that the average was nearer to 85 per cent.

We did not extract our dried tissue with ether before ashing it for various reasons. It is possible that the ash of tissue extracted with perfectly dry ether more nearly represents the inorganic salt content of the tissue than in the case where the ether extraction is omitted. It would be very interesting to determine the differences between the ashes of extracted and unextracted tissue and we hope to take up this question later on. We think it is important, however, as a preliminary step to have total ash determinations for smooth muscle, which should be comparable, as far as possible, to the ash determinations which have already been made for the striated muscle of the same animals.

In making our determinations we used in general the methods employed

by Katz,⁹ and we carried along with each pair of determinations on smooth muscle a similar pair on striated muscle. Our methods were exactly similar to those of Katz in the determinations of potassium, sodium, iron, calcium, and magnesium.

We determined the phosphorus in both striated and smooth muscle as Katz did in three portions, extracting our tissue first with boiling water, and then with 95 per cent alcohol in the Soxhlet apparatus. The phosphorus was determined in the water extract, in the alcoholic extract, and in the residue which had been extracted with both water and alcohol. In the striated muscle we found, as Katz did, that a little over 80 per cent of the phosphorus appeared in the water extract. In the case of smooth muscle, on the other hand, the phosphorus which appeared in the water extract was a little under 70 per cent of the total. Our figures for smooth muscle are given in the experimental protocols. We have not put these figures in our tables, because we think (as, indeed, Katz admits) that they are far from correct quantitatively. Our water extracts from smooth muscle were quite opalescent, and left, on evaporation, considerable quantities of greasy material which might well have been lipoid. The results indicate that smooth muscle contains a good deal more non-diffusible phosphorus than striated muscle, but they require further elucidation.

In analyzing for phosphorus we fused our residues with sodium hydroxide and potassium nitrate in silver dishes instead of ashing them in platinum crucibles as Katz did.

In the case of chlorine a preliminary determination was made by the method employed by Katz, in this determination the chlorine was found to amount to 0.0988 per cent of the fresh tissue. We were not entirely satisfied with this determination and made two others, in which the fresh tissue was fused with sodium hydroxide and potassium nitrate and analyzed for chlorine by the Volhard-Arnold method described by Hawk.¹⁰ In these two experiments the chlorine was found to be 0.1200 per cent and 0.1191 per cent of the weight of the fresh tissue respectively.

The sulphur was determined by the method described by Hawk on pp 381-383 of the work just quoted. The tissue was fused with sodium hydroxide and potassium nitrate in a silver dish over an alcohol flame, and the sulphates were subsequently precipitated by means of barium chloride.

Striated muscle analyzed by these methods for phosphorus, chlorine, and sulphur gave results not far from those obtained by Katz (see tables).

Our results are given in Table II.

⁹ Katz *Arch f d ges Physiol*, LVIII, p 1, 1896.

¹⁰ Hawk *Practical Physiological Chemistry*, 3d Edition, Philadelphia pp 390 and 391, 1910.

TABLE II
 Constituents of frog's muscle given as percentages of the fresh tissue

	K	Na	Fe	Ca	Mg	P	Cl	S	SOLIDS	WATER
<i>Striated muscle (Rana Catesbiana and Rana Esculentia)</i>										
Our figures for Rana Catesbiana Katz's Averages for Rana Esculentia	0 3183	0 0572	0 0099	0 0335	0 0288	0 1551	0 0650	0 1402	20 01	79 99
	0 3518	0 0500	0 0091	0 0228	0 0313	0 1511	0 0674	0 1322	20 20	79 74
	0 3500	0 0536	0 0096	0 0281	0 0300	0 1517	0 0662	0 1407	20 13	79 87
	0 3080	0 0552	0 0062	0 0157	0 0235	0 1802	0 0102	0 1033	18 38	81 62
<i>Smooth muscle (stomach muscle of Rana Catesbiana)</i>										
Our figures I II Averages	0 3063	0 0618	0 0007	0 0012	0 0132	0 1460	0 1191	0 1721	17 39	82 61
	0 3137	0 0801	0 0007	0 0012	0 0126	0 1281	0 1200	0 1501	17 99	82 01
	0 3250	0 0720	0 0007	0 0012	0 0129	0 1372	0 1195	0 1612	17 70	82 30

THE RELATION OF OUR RESULTS TO THOSE OF SAIKI, MACALLUM AND
COSTANTINO

We agree with Macallum and Costantino and differ from Saiki in finding the sodium and potassium content of smooth muscle not widely different from that of striated muscle. We find in general that the ash of smooth muscle is much more nearly like that of striated muscle than Saiki's figures would indicate, our figures for potassium, magnesium, phosphorus, and sulphur are much higher, and our figures for sodium, calcium, and chlorine, much lower than his. Saiki lays some emphasis on the large amount of calcium which he found in smooth muscle, and suggests (pp 492 and 493) that this element may have some connection with the well-known power of smooth muscle to remain for a long time in a state of marked tonic contraction. Our figure for calcium is only about one-eighth of that of Saiki, and only about one-fourth of what Katz found for the striated muscle of the frog. We are not inclined to attribute very much significance to the percentage of calcium found in either striated or smooth muscle. In Katz's results this element is by far the most variable of all, ranging from 0.002 per cent in the steer to 0.018 per cent in the rabbit among mammals, and rising to nearly 0.04 per cent in the eel and pike. Saiki's figures seem to show that the amount of calcium varies widely even in the smooth muscle of the same animal, they range from 0.022 per cent to 0.042 per cent.

Our figure for iron is very low, which indicates that our tissue contained very little blood. We were careful to dissect off the larger blood vessels from the outside of the stomach, and our method of pressing our sheets of muscle against filter paper probably freed them from blood quite effectually. The gross and microscopic appearances of our tissue agreed in indicating that there was very little blood in it.

Our results show that smooth muscle contains about twice as much chlorine as striated muscle, and in this we are in general agreement with both Saiki and Costantino. And we agree with Saiki, Costantino, and Macallum in finding more sodium in smooth muscle than in the striated muscle of the same animal, though we do not think the difference is so marked as Saiki's figures would indicate. Histological examination has convinced us that our

smooth muscle contained a larger proportion of muscle fiber than do most samples of striated muscle, we therefore think that all the work done so far on smooth muscle shows that the fibers of this tissue contain considerably more sodium and chlorine than do those of striated muscle. It is interesting to note that the amounts of sodium and chlorine which we find in smooth muscle are about such as unite to form sodium chloride.

GENERAL DISCUSSION

There is reason to believe that a large proportion of the sodium and chlorine of the blood plasma and lymph, and of the potassium and phosphorus of the striated muscle fibers exists as diffusible salt, and it is rather generally supposed that semi-permeable membranes surround the muscle fibers and prevent the inter-diffusion of the sodium chloride and potassium phosphate. Overton¹¹ has collected a good deal of evidence which tends to prove that the muscle fibers are surrounded by such semi-permeable membranes, he believes that such surrounding membranes constitute a very general peculiarity of both animal and vegetable cells. On the hypothesis that such membranes exist there have already been founded theories of stimulation and of anesthesia, and it is altogether probable that many of the nutritional processes are controlled by the nature of the bounding surfaces between cells and the surrounding lymph.

Overton's work makes it seem probable that the fibers of striated muscle are surrounded by membranes permeable to water and to fat solvents, and impermeable to sugars and inorganic salts. But the hypothesis that such membranes exist is, as Overton acknowledges, far from explaining all the known facts. The question arises, how growing muscle fibers get their supply of potassium phosphate. The concentration of potassium and phosphorus in the blood plasma and lymph is very low, and salts do not diffuse from a region of lower to one of higher concentration. The difficulty can be overcome only by supposing that the potassium and phosphorus enter the muscle fibers not as potassium phosphate but in some other probably organic combination or combinations.

¹¹ Overton *Arch f ges Physiol*, xcii pp 115 and 346, 1902, cv, p 207, 1904

It is highly improbable that this process occurs only in growing muscle fibers. The existence of a membrane absolutely impermeable to salts is hardly conceivable, and it is not difficult to find experimental evidence for the view that potassium phosphate escapes from the muscle fibers quite readily. The work of Urano¹² and of Fahr¹³ shows that the slightest injury to an excised muscle causes a large escape of potassium and phosphorus from its fibers into a surrounding isotonic sugar solution. Considerable quantities of these elements may be lost without impairing the muscle's irritability. Further, Klug and Olsavsky¹⁴ have shown that muscular work causes an increase in the phosphorus excreted in the urine.

It is, therefore, not a tenable hypothesis that the "semi-permeable membranes" surrounding living cells are absolutely impermeable to inorganic salts. The impermeability must be regarded as relative, and the solution of the most interesting physiological problems will depend on our knowledge of the degree and variability of this relative impermeability. In the case of striated muscle, there can be little doubt that under normal circumstances there is a frequent, if not continual, loss of potassium phosphate by the fibers, and this loss must be compensated by their taking these elements from the lymph in some other combination. It is generally believed that the striated muscle fibers contain a certain amount of phosphorus in organic combination with lecithin and nuclein, and it has been shown by Katz¹⁵ that the phosphorus from these sources appears in the ash of the tissue.

Overton lays a good deal of stress on the fact that the intake of water by striated muscle from hypotonic solutions is smaller than it should be on the supposition that all of the water within the muscle fibers acts as a solvent for the muscle salts. To explain this he has introduced the hypothesis that a part of the water within the muscle fibers is held in a sort of chemical combination by the muscle colloids and thus prevented from acting as a solvent for the salts. This hypothetical organically combined water he calls "Quellungswasser," the term will be translated in this article by the phrase *organic water*.

¹² Urano *Zeitschr f Biol*, 1, p 212, 1907, 1, p 483, 1908

¹³ Fahr *Ibid*, 11, p 72, 1908

¹⁴ Klug and Olsavsky *Arch f d ges Physiol* liv, p 21, 1893

¹⁵ *Loc cit*

The evidence which Overton has accumulated makes the existence of semi-permeable membranes very probable in the case of striated muscle, but we wish to point out that the fact that the ash of a tissue is different from that of the lymph is not of itself sufficient to show that the cells or fibers of that tissue are surrounded by semi-permeable membranes. To explain the conditions existing in smooth muscle, for instance, without invoking the aid of semi-permeable membranes, it is only necessary to suppose that the potassium, phosphorus, magnesium, and sulphur of the ash exist in the living tissue in a non-diffusible form, and to extend somewhat the conception of organic water which Overton himself has introduced. For, if a little more than half of the water of the tissue existed as organic water, the percentages of sodium and chlorine given in the tables would be sufficient to make the concentration of these elements in the remaining inorganic water the same as they have in the lymph.

There are reasons for believing that the fibers of smooth muscle are not surrounded by semi-permeable membranes. For example, a great many facts indicate that these fibers lose fluid when they contract,¹⁶ and it is difficult to see how this could occur if they were separated from their surroundings by membranes impermeable to inorganic salts. Further, the changes of weight undergone by smooth muscle in various solutions of sugars and salts are so different from those undergone by striated muscle in the same solutions that it is difficult to believe that the two sets of phenomena have anything in common. Some of these peculiarities in the behavior of smooth muscle have been already reported,¹⁷ others have been experimentally determined by one of us and may be briefly described here.

Both smooth and striated muscle gain in weight if they are immersed in a half strength Ringer solution.¹⁸ If the rates of gain be determined at short (five to ten minutes) successive intervals and the results plotted as curves, it will be found that the curves in the two cases have entirely different characters. Striated

¹⁶ Meigs *Amer Journ of Physiol*, **xxii**, p 477, 1908, **xxix**, p 317, 1912

¹⁷ Meigs *Ibid*, **xxvii**, p **xxvii**, 1911

¹⁸ By a half-strength Ringer solution is meant a solution with the following formula, NaCl, 0.32 gram, KCl, 0.01 gram, CaCl₂, 0.012 gram, NaHCO₃, 0.01 gram, H₂O 100 grams

muscle gains most rapidly in the first five minutes and less rapidly in each succeeding period, the curve of gain in weight is, at least in the early stages, concave to the abscissa and of such a character as to suggest that the water intake may be the result of osmosis. Smooth muscle, on the other hand, gains no more rapidly in the first five minutes than in the next four or five succeeding equal periods. Indeed, the gain may be slower at first so that the curve becomes slightly convex to the abscissa, but it tends in general to take the form of a straight line.

Striated muscle maintains its original weight for many hours in a 7.5 per cent cane sugar solution, while smooth muscle may nearly double its weight in this solution in the course of an hour. But, if nine parts of the sugar solution are mixed with one part of Ringer's solution, the smooth muscle shows no marked tendency to gain weight in the mixture. These facts are opposed to the view that the smooth muscle fibers are surrounded by semi-permeable membranes. The mixture of sugar solution with Ringer solution has nearly the same osmotic pressure as the sugar solution itself, yet the muscle gains weight rapidly in this and fails to gain in the mixture. The behavior of the muscle in these solutions, therefore, bears no relation to their osmotic pressure.

It must be added that both striated and smooth muscle live for twenty-four hours or more in half strength Ringer, in 7.5 per cent cane sugar solution, and in mixtures of the sugar solution with Ringer at room temperature. The changes of weight undergone by the tissues in these media must therefore be regarded as the reactions of living tissue.

We have some very incomplete evidence to show that the potassium and phosphorus of smooth muscle is present in the living tissue in a non-diffusible form, and that smooth muscle contains more lipoid than striated muscle. We have cut pieces of smooth muscle across the fibers, kept them for several hours in isotonic saccharose solution, and then compared their potassium content with that of fresh muscle used as a control. We find that very little (less than 5 per cent) of the tissue's potassium diffuses out into the sugar solution under these conditions.

The boiling water extracts from smooth muscle contain a considerably less proportion of the tissue's total phosphorus than do similar extracts from striated muscle (see ante, p. 406, and experi-

mental protocols for phosphorus) though the smooth muscle extracts contain a larger proportion of solid matter Both of these facts are evidence for the view that a large proportion of the smooth muscle phosphorus comes from the lipoid of the tissue, and is therefore non-diffusible under normal conditions

The facts which are known at present, then, point to the following conclusions in regard to smooth muscle

1 The fibers of this tissue are not surrounded by semi-permeable membranes

2 Most of the water of the smooth muscle fibers is held by the colloids of the living tissue as organic water

3 Most of the potassium, phosphorus, sulphur, and magnesium, which appear in the ash of smooth muscle, are present in the living tissue in a non-diffusible form

PROTOCOLS OF THE EXPERIMENTS ON SMOOTH MUSCLE

Potassium and Sodium I 6.787 grams fresh smooth muscle yielded 0.0508 gram combined KCl and NaCl and 0.1299 gram K_2PtCl_6

0.1299 gram K_2PtCl_6 is equivalent to 0.0397 gram KCl

0.0508 gram - 0.0397 gram = 0.0111 gram, quantity of NaCl

0.0397 gram KCl contains 0.0203 gram K 0.0111 gram NaCl contains 0.0044 gram Na

6.787 gram muscle, therefore, contains 0.0203 gram or 0.3063 per cent K and 0.0044 gram or 0.0648 per cent Na

II 6.3422 gram fresh smooth muscle yielded 0.0546 gram combined KCl and NaCl, and 0.1364 gram K_2PtCl_6

0.1364 gram K_2PtCl_6 is equivalent to 0.0417 gram KCl

0.0546 gram - 0.0417 gram = 0.0129 gram, quantity of NaCl

0.0417 gram KCl contains 0.0218 gram K 0.0129 gram NaCl contains 0.0051 gram Na

6.3422 gram muscle contains, therefore, 0.0218 gram or 0.3437 per cent K and 0.0051 gram or 0.0804 per cent Na

Iron I 15.2106 grams fresh smooth muscle yielded 0.0003 gram $Fe_2(PO_4)_2$
= 0.0001 gram Fe = 0.0007 per cent Fe

II 15.1614 grams fresh smooth muscle yielded 0.0003 gram $Fe_2(PO_4)_2$ = 0.0001 gram Fe = 0.0007 per cent Fe

Calcium I 15.2106 gram fresh smooth muscle yielded 0.0009 gram CaO
= 0.0006 gram Ca = 0.0042 per cent Ca

II 15.1614 grams fresh smooth muscle yielded 0.0009 gram CaO = 0.0006 gram Ca = 0.0042 per cent Ca

Magnesium I 15.2106 grams fresh smooth muscle yielded 0.0093 gram $Mg_2P_2O_7$ = 0.0020 gram Mg = 0.0132 per cent Mg

II 15 1614 grams fresh smooth muscle yielded 0 0089 gram $\text{Mg}\cdot\text{P}\cdot\text{O}_7$ = 0 0019 gram Mg = 0 0126 per cent Mg

Phosphorus I The water extract from 10 8756 grams fresh smooth muscle yielded 0 0374 gram $\text{Mg}\cdot\text{P}_2\text{O}_7$ = 0 0104 gram P = 0 0958 parts P per 100 parts muscle

The alcohol extract from 10 8756 gram smooth muscle yielded 0 0139 gram $\text{Mg}_2\text{P}_2\text{O}_7$ = 0 0039 gram P = 0 0356 parts P per 100 parts muscle

The residue from 10 8756 grams smooth muscle which had been extracted with water and alcohol yielded 0 0057 gram $\text{Mg}\cdot\text{P}_2\text{O}_7$ = 0 0016 gram P = 0 0146 parts P per 100 parts muscle

This sample of smooth muscle, therefore, contained in all 0 0958 + 0 0356 + 0 0146 or 0 1460 per cent P

II The water extract from 10 3912 grams fresh smooth muscle yielded 0 0343 gram $\text{Mg}\cdot\text{P}_2\text{O}_7$ = 0 0096 gram P = 0 0919 parts P per 100 parts muscle

The alcohol extract from 10 3912 gram smooth muscle yielded 0 0097 gram $\text{Mg}_2\text{P}_2\text{O}_7$ = 0 0027 gram P = 0 0260 parts P per 100 parts muscle

The residue from 10 3912 grams smooth muscle, which had been extracted with water and alcohol, yielded 0 0039 gram $\text{Mg}\cdot\text{P}_2\text{O}_7$ = 0 0011 gram P = 0 0105 parts P per 100 parts muscle

This sample of smooth muscle, therefore, contained in all 0 0919 + 0 0260 + 0 0105 or 0 1284 per cent P

Chlorine I 3 5270 grams fresh smooth muscle yielded 0 0169 gram AgCl = 0 0042 gram Cl = 0 1191 per cent Cl

II 3 5008 grams fresh smooth muscle yielded 0 0169 gram AgCl = 0 0042 gram Cl = 0 1200 per cent Cl

Sulphur I 6 3489 gram fresh smooth muscle yielded 0 0797 gram BaSO_4 = 0 0109 gram S = 0 1724 per cent S

II 6 0210 grams fresh smooth muscle yielded 0 0658 gram BaSO_4 = 0 0090 gram S = 0 1501 per cent S

The experiments described in these protocols are those of which the results are given in Table II We have analyzed other samples of smooth muscle for potassium, sodium, phosphorus, and chlorine, but have not used the results of these analyses in calculating our averages, chiefly because they were preliminary single analyses without accompanying parallels The results were, however, not far from those which appear in the table and may be given here Potassium, 0 3458 per cent, sodium, 0 0506 per cent, phosphorus, 0 1494 per cent, chlorine, 0 0988 per cent

The percentages of water and total solids in our samples of tissue were determined by drying them at between 100° and 110°C until they reached constant weight This usually required between twenty-four and seventy-two hours, though the loss of weight after twenty-four hours was very slight The figures in the table represent the widest differences found in six determinations, and the average is calculated from all six In the four determinations not given in the table the total solids were found to be 17 49 per cent, 17 60 per cent, 17 82 per cent, and 17 93 per cent respectively

THE TOXICITY OF SUGAR SOLUTIONS UPON FUNDULUS AND THE APPARENT ANTAGONISM BETWEEN SALTS AND SUGAR.

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(Received for publication, March 26, 1912)

In 1901 the writer pointed out the fundamental difference in the behavior of electrolytes and non-conductors in regard to antagonistic action on the eggs of *Fundulus* ¹ While it was to a large extent possible to antagonize the toxic action of one electrolyte by another it was impossible to antagonize the toxic action of an electrolyte by a non-electrolyte Gies and I found an apparent exception to this rule in the case of the salts of heavy metals, *e g* , ZnSO_4 , which could be antagonized by an excess of cane sugar,² and Sumner found afterwards that the toxic action of CuSO_4 could also be deferred through the addition of cane sugar ³ We were inclined to explain this apparent exception on the assumption of the formation of saccharates with a diminution in the concentration of the free metal ions In the case of the antagonization of one salt by another we are dealing with a common action of both salts upon one or several colloids on the surface of the organism ⁴

The writer recently made experiments on the toxic action of sugars on *Fundulus*, the results of which, at first sight, seemed to speak in favor of the possibility of an antagonization of the toxic action of sugars by salts A more thorough analysis, however, showed that in this case the real antagonism was between two elec-

¹ *Amer Journ of Physiol* , vi, p 411, 1902

² *Pflüger's Archiv* , xciii, p 246, 1902

³ *Amer Journ of Physiol* , vii, p 61, 1907

⁴ *Science*, xxxiv, p 653, 1911

trolytes The method employed was identical with that used in the previous experiments by Mr Wasteneys and the writer A series of sugar solutions was prepared, 500 cc of each, and six *Fundulus* were put into each of these solutions, after having been washed repeatedly in fresh and distilled water The number of fish which had survived the treatment was ascertained daily

Tables I and II give the records of two simultaneous experiments, one with various concentrations of cane sugar alone, the other with the same concentrations of cane sugar solutions made up in a $\frac{M}{8}$ solution of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ (in the usual proportion) instead of in water

A comparison of the two tables gives the typical picture of an antagonistic action, while in the pure cane sugar solutions all the fish were dead on the seventh day, in the solutions of cane sugar

TABLE I

Number of surviving fish in various concentrations of cane sugar in water

AFTER DAYS	CONCENTRATION OF CANE SUGAR							
	$\frac{M}{128}$	$\frac{M}{64}$	$\frac{M}{32}$	$\frac{M}{16}$	$\frac{M}{8}$	$\frac{M}{4}$	$\frac{3M}{8}$	$\frac{M}{2}$
1	4	4	4	6	6	4	6	5
3	4	2	3	6	6	3	1	0
5	2	0	2	1	0	0	0	
7	0		0	0				

TABLE II

Number of surviving fish in various concentrations of cane sugar in $\frac{M}{8}$ NaCl, KCl, CaCl.

AFTER DAYS	CONCENTRATION OF CANE SUGAR							
	$\frac{M}{128}$	$\frac{M}{64}$	$\frac{M}{32}$	$\frac{M}{16}$	$\frac{M}{8}$	$\frac{M}{4}$	$\frac{3M}{8}$	$\frac{M}{2}$
1	6	6	6	6	6	6	6	5
3	6	6	6	6	6	4	4	0
5	6	6	6	6	6	0	0	
7	6	6	5	6	4			
9	6	5	3	0	0			
11	6	5	3					
13	5	0	1					

made up in $\frac{M}{8}$ NaCl + KCl + CaCl₂ practically all the fish were alive in all the sugar solutions below $\frac{M}{4}$. Nevertheless the salt did not antagonize the toxic action of the sugar solutions in this case, but the action of a fermentation product from the sugar, namely an acid. In previous publications by Mr Wasteneys and the writer it was shown, that the toxic effects of acid on *Fundulus* could be antagonized by salts, and that the concentration of acid the toxic action of which can be antagonized, is not inconsiderable.⁵ Table III gives the maximum concentration of acid which is antagonized by a mixture of NaCl + KCl + CaCl₂ of various concentration

TABLE III

CONCENTRATION OF THE MIXTURE OF NaCl KCl AND CaCl ₂	MAXIMAL QUANTITY OF $\frac{N}{10}$ HCl WHICH THE FISH WILL RESIST IN THESE SOLUTIONS
	cc
0	0 1-0 2
$\frac{M}{100}$	0 2
$\frac{M}{10}$	0 5
$\frac{M}{5}$	1 0
$\frac{M}{2}$	1 2-1 4
$\frac{M}{1}$	0 8
$\frac{M}{2}$	0 6
$\frac{M}{3}$	0 3
$\frac{M}{4}$	0 2

The sugar solutions with and without salt soon become turbid and later almost opaque and an examination showed that the solution was teeming with bacteria. A titration of the sugar solutions with NaOH gave a rather high degree of acidity as was to be expected. Twenty-five cubic centimeters of the $\frac{M}{32}$ cane sugar solution in $\frac{M}{8}$ NaCl + KCl + CaCl₂ required on the ninth day 7.5 cc $\frac{N}{100}$ NaOH for neutralization. The toxic action of the sugar solution on the fish was therefore due in part not to the sugar but to a fermentation product, namely an acid. That the fish died of acid poisoning was also indicated by their external appearance. As I pointed out in a previous paper the epidermis

⁵ *Biochem Zeitschr*, xxviii, p 489, 1911, xxxix, p 167, 1912

becomes white in the case of acid poisoning, and the fish which died in the sugar solutions became white before death occurred

Similar results were obtained in experiments with dextrose, as Tables IV and V will indicate. The experiments represented in these two tables were made simultaneously

TABLE IV

Number of surviving fish in various concentrations of dextrose in water

AFTER DAYS	CONCENTRATION OF DEXTROSE									
	$\frac{M}{128}$	$\frac{M}{64}$	$\frac{M}{32}$	$\frac{M}{16}$	$\frac{M}{8}$	$\frac{M}{4}$	$\frac{3M}{8}$	$\frac{M}{2}$	$\frac{3M}{4}$	M
1	6	6	6	6	6	6	6	6	0	0
3	5	4	4	4	0	0	0	0		
4	0	0	0	0						

TABLE V

Number of surviving fish in various concentrations of dextrose in $\frac{M}{8}$ NaCl + KCl + CaCl₂

AFTER DAYS	CONCENTRATION OF DEXTROSE									
	$\frac{M}{128}$	$\frac{M}{64}$	$\frac{M}{32}$	$\frac{M}{16}$	$\frac{M}{8}$	$\frac{M}{4}$	$\frac{3M}{8}$	$\frac{M}{2}$	$\frac{3M}{4}$	M
1	6	6	6	6	6	6	6	6	6	0
3	6	6	6	6	6	6	6	2	0	
4	5	6	6	6	5	6	6	0		
5	2	6	4	2	5	5	3			
7	0	0	0	0	0	0	0			

The dextrose solutions were less toxic when they were made up in $\frac{M}{8}$ Ringer solutions than when made up in H₂O. As was to be expected the dextrose solutions soon became turbid and opaque and contained a considerable amount of free acid. Again the inference was unavoidable that the antagonism in this case existed between the free acid and the salt and not between the sugar and the salt, and this surmise was supported by the fact that the epidermis of the fish showed the whiteness characteristic for the effect of acid. This idea that the acid and not the sugar solution killed the fish could be put to a further test by a comparison of the toxic action of sugar solutions which were allowed to ferment and sugar

solutions which were renewed sufficiently often to prevent a high concentration of acid through the action of bacteria. Into each of two dishes with 500 cc $\frac{N}{8}$ dextrose six *Fundulus* were put. The one dish remained unaltered, the fish of the other dish were transferred into a fresh $\frac{N}{8}$ dextrose solution every twenty-four hours. In these latter solutions fermentation began also and some acid was formed, but this solution remained clear and the amount of acid was too small to do much harm. Care was taken that the new sugar solutions always had the same temperature as the old ones. In the dextrose solution which was not changed all the fish were dead after four days, as in the previous experiments, and they died with the symptoms of acid poisoning. The six fish which were transferred into a fresh $\frac{N}{8}$ dextrose solution every day are still alive and apparently normal today, on the twenty-sixth day of the experiment. This experiment was repeated with the same result.

A similar experiment was started with an $\frac{N}{8}$ solution of cane sugar. Four dishes, each with 500 cc $\frac{N}{8}$ cane sugar, were prepared and six *Fundulus* put into each dish. In two dishes the solution was not renewed, the fish from the other two solutions were transferred every day into a fresh $\frac{N}{8}$ solutions of cane sugar.

In the dishes in which the cane sugar solution was not renewed the fish were all dead after three and five days respectively. The fish which were transferred every day are partly alive today after fifteen days, in one dish five out of the six put there originally are still alive and two in the other dish.

These experiments leave no doubt that at least part of the toxic effects of the sugar solutions is due to the formation of acid and that the antagonism expressed in Tables I and II and IV and V is the antagonism between acid and salts.

It became a matter of interest to find out to what extent a pure sugar solution may be called toxic for these fish. For this purpose six fish each were put into a $\frac{N}{16}$, $\frac{N}{8}$, $\frac{N}{4}$ and $\frac{N}{2}$ solution of cane sugar and the solution renewed each day. Table VI gives the results.

The rapid death in the $\frac{N}{2}$ and $\frac{N}{4}$ cane sugar solution cannot be ascribed to a product of fermentation, e g, acid, here we are possibly dealing with a direct action of the sugar. The $\frac{N}{8}$ and $\frac{N}{16}$ solutions, however, behave almost like an indifferent salt-free solution.

TABLE VI

Number of surviving fish in various concentrations of cane sugar

AFTER DAYS	CONCENTRATION OF CANE SUGAR			
	$\frac{M}{16}$	$\frac{M}{8}$	$\frac{M}{4}$	$\frac{M}{2}$
1	6	6	6	6
2	6	6	5	4
3	5	6	5	1
4	5	6	4	0
7	4	5	1	
9	4	4	0	
11	4	4		

The writer is not in a position to judge whether or not these results can be applied to the interpretation of the symptoms of patients with glycaemia. During the experiments the fish were not fed and therefore did not take up any salts.

SUMMARY OF RESULTS

1 *Fundulus* live longer in solutions of cane sugar and dextrose made up in a $\frac{M}{8}$ solution of $\text{NaCl} + \text{KCl} + \text{CaCl}_2$ than in the same sugar solution without salts.

2 It is shown that in these solutions (through bacterial action) a considerable amount of acid is formed and that the apparent antagonism between sugar and salt is in reality a case of antagonism between acid and salt.

3 This conclusion is corroborated by the observation that $\frac{M}{8}$ solutions of dextrose or cane sugar, which kill the fish in a few days become almost harmless if the solution is renewed every twenty-four hours, whereby the multiplication of bacteria and the formation of acid is considerably diminished.

4 These experiments also show that $\frac{M}{8}$ or weaker solutions are in themselves nearly harmless and that their apparent toxicity upon *Fundulus* is due to the formation of acid or other products under the influence of bacteria. Concentrations of sugar equal to or greater than $\frac{M}{8}$ are, however, harmful independently of the acid formation by bacteria.

CARBOHYDRATE ESTERS OF THE HIGHER FATTY ACIDS

III MANNITE ESTERS OF LAURIC ACID

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In the earlier papers of this series the preparation and chemical and physiological properties of some mannite esters of stearic acid have been described. These compounds, because of their high melting point and low digestibility were not well adapted to physiological investigations, and it was decided to prepare similar compounds of a fatty acid lower in the series, in the hope that they would prove more suitable. Lauric acid was chosen as being of sufficiently lower carbon content to give a decided difference in properties, and because its wide distribution promised a ready supply of material. To prepare lauric acid, laurel oil was saponified and the fatty acids fractionally distilled at low pressure according to the directions of Krafft¹. The fraction so obtained, which boiled at 225° under 100 mm pressure, was found to have too low a melting point. It was, therefore, recrystallized from ice cold alcohol until a product was obtained which melted at 42°C and distilled at 225° under 100 mm pressure. This sample was used in the preparation of the mannite esters described below.

MANNITE DILAURATE

Mannitan dilaurate was prepared from the lauric acid as follows

Ten grams of mannite (Kahlbaum) were dissolved in 200 cc warm (38°) concentrated sulphuric acid and 23 grams lauric acid stirred in. When all

¹ Krafft *Ber d deutsch chem Gesellsch*, 1911, p 4344, 1903

had dissolved the mixture was digested over night in an incubator at 38°C. To separate the mannite esters from the acid mother liquor, the solution was poured with constant stirring into iced, saturated ammonium sulphate, let stand for a short time, transferred to a filter, let drain as completely as possible, then washed once with saturated ammonium sulphate. After again draining, the mass was transferred to boiling alcohol in which it separated into two layers, a lower, watery layer containing most of the ammonium sulphate and acid, and an upper alcoholic layer which contained the mannite ester. The lower layer was siphoned off and the alcoholic solution allowed to cool. The ester was filtered off and recrystallized from alcohol until the melting point was constant.

The yield from the second recrystallization from alcohol was 13 grams, corresponding to about 45 per cent of the lauric acid used—a very much lower yield than was obtained from stearic acid. Grun in his work on the synthetic fat has also found that the sulphuric acid synthesis becomes less effective the smaller the molecular weight of the fatty acid.²

The product obtained greatly resembles the homologous stearic acid compound. It separates from alcohol in microscopic needles which when dried are snow white. It is practically insoluble in cold alcohol, and only slightly soluble in cold ether, benzol or chloroform. It dissolves in these solvents when heated to boiling and separates on cooling in crystalline form. It is heavier than water. Melting point (uncorrected), 122°C.

It is slightly dextrorotatory. Because of its slight solubility there was the same difficulty in making the polarimetric reading as with the stearic acid ester.

One gram of mannitan dilaurate dissolved in 25 cc. chloroform at 50°C and read in a 1 dm. tube gave a reading of +0.34°.

$$[\alpha]_D^{20} = +8.5^{\circ}$$

ANALYSIS A fatty acid determination was made in the regular way. The ester dissolved in hot alcohol was saponified by alcoholic alkali, most of the alcohol driven off and the residue taken up with water. The fatty acid set free by the addition of sulphuric acid was filtered, washed with hot water until the wash water showed no trace of sulphate, then dried and weighed.

(1) 0.5938 gram mannitan dilaurate yielded 0.7218 gram lauric acid = 75.68 per cent.

(2) 1.0449 gram mannitan dilaurate yielded 0.7920 gram lauric acid = 75.79 per cent.

Calculated for mannitan dilaurate, $C_8H_{10}O_3(C_{11}H_{23}COO)_2$ = 75.75 per cent.

- COMBUSTION (1) 0.1512 gram yielded 0.3773 gram CO_2 and 0.1424 gram H_2O
 (2) 0.1496 gram yielded 0.3727 gram CO and 0.1423 gram H_2O
 (3) 0.1387 gram yielded 0.3452 gram CO

	Calculated	1	Found 2	3
C	63.18	63.06	67.95	67.88
H	10.61	10.47	10.57	

Mannitan dilaurate, because of its high melting point, did not seem promising, and no feeding experiments were made with it.

ISOMANNID DILAURATE

Isomannid dilaurate was prepared from the mannitan dilaurate by a short heating to 200°C in the same way as isomannid distearate was prepared from mannitan distearate.³ The product was freed from small portions of the unchanged mannitan ester by repeated treatment with cold ether, filtering and evaporating to dryness, until it dissolved clear in a small portion of the cold solvent. It was then taken up with ether, titrated with alcoholic alkali and phenolphthalein to remove any free lauric acid, filtered, treated with bone black to remove color, again filtered and evaporated to dryness. The pure white product was further purified by several recrystallizations from ice cold alcohol.

The product is snow-white and when melted and cooled appears crystalline. It is lighter than water and emulsified readily with warm soap solution.

When heated above 100°C it slowly volatilizes with decomposition. It is readily soluble in cold ether, benzol or chloroform and is quite soluble in cold alcohol.

Its melting point is 37.5°C (uncorrected).

Optical activity Like the corresponding stearic acid ester it is strongly dextrorotatory. Determinations were made in ether and benzol solution.

ETHER 0.343 gram in 50 cc ether in 2 dm tube gives reading $+1.73$,
 $\approx +125.5^\circ$
 0.600 gram in 16 cc ether in 1 dm tube gives reading $+4.69$,
 $\approx +125.1^\circ$

³ Bloor *This Journal*, **4**, p. 141, 1912

BENZOL 0.494 gram in 50 cc benzol in 2 dm tube gives reading +4.95,
= +125.0

Refraction Abbe-Zeiss refractometer

$n_{40} = 1.4570$ $n_{46} = 1.4555$ $n_{50} = 1.4535$ $n_{60} = 1.4500$

Its low melting point and ready saponification rendered isomannid dilaurate a promising substance from a physiological point of view, and experiments were conducted to determine its availability for the animal organism. Because of the difficulty of obtaining pure lauric acid in large quantity, and since for physiological purposes a pure compound was not required, a mixture of isomannid esters of lauric, myristic, etc., acids was used. The acids were obtained from cocoanut oil, which consists mainly of esters of capric (20 per cent), lauric (40 per cent) and myristic (24 per cent) acids together with small amounts of palmitic (10 per cent) and oleic (5 per cent) acids. The palmitic and oleic acids and most of the capric acid were removed as follows:

Commercial cocoanut oil was saponified with alcoholic potash. The soaps were dissolved in water, boiled, skimmed free from unsaponified material, and the fatty acids set free with sulphuric acid. After washing several times with hot water, then cooling, the cake of fatty acids was dried and dissolved in just sufficient alcohol to avoid any separation when cold. Enough saturated magnesium acetate solution was added to precipitate the palmitic acid, the mixture set in the ice chest over night, then filtered on a Buchner filter. The precipitate contained the palmitic acid. For the separation of the oleic acid, recourse was had to the solubility of its lead soap in ether. To the filtrate from the magnesium precipitate a hot saturated water solution of lead acetate was added as long as a precipitate was formed, and the mixture was set in the cold over night. The lead soaps were filtered off, pressed as dry as possible, then boiled out with water. They melted and sank to the bottom, and on cooling solidified. The water was poured off, the solid mass dried, melted to get rid of the remaining water, shaved fine and extracted several times with cold ether. The insoluble residue was boiled out with dilute hydrochloric acid sufficient to remove the lead, washed well with boiling water and finally cooled.

The mixture of fatty acids so prepared, consisting largely of lauric acid, was used in the preparation of the mannite esters for the feeding experiments. The proportions of the fatty acids varied somewhat, depending on the sample of oil used and on the conditions of the separation, so that ester mixtures prepared from differ-

ent samples differed to some extent in melting point and optical activity. Isomannid esters were prepared from these fatty acid mixtures by the method outlined above.

Weighed quantities of the esters together with meat were fed to animals, and the amount of digestion determined by the optical activity of the ether extract of the feces. It was obviously necessary to know the optical activity of the ether extract of normal feces under parallel conditions. Some preliminary experiments were, therefore, carried out in which the animal was fed lean meat and lard.

PRELIMINARY (CONTROL) EXPERIMENTS

A cat, weighing 1.6 kilos, was starved for two days, then fed on each of three days, 75 grams of hashed lean beef, 7.5 grams lard and 3 grams bone ash (added in order that the resulting feces could be readily dried and powdered for extraction). The feces were collected, dried, broken up and extracted three to four hours with ether in a Soxhlet extractor. The ether extract was filtered, evaporated to 50 cc and readings taken in a polariscope using a 1 dm tube.

First day—reading of 50 cc ether extract of feces in 1 dm tube = $+0.04^\circ$

Second day—reading of 50 cc ether extract of feces in 1 dm tube = $+0.07^\circ$

Third day—reading of 50 cc ether extract of feces in 1 dm tube = $+0.03^\circ$

Average reading for one day's feces on above diet = $+0.05^\circ$

Other blank experiments were carried out in the intervals of the ester experiments with results substantially the same.

FEEDING EXPERIMENTS WITH THE ISOMANNID ESTERS

The mixture of esters used in these experiments had a melting point of 25° and specific rotation of $+87.0^\circ$.

Between each of the experiments came two days of feeding of meat mixed with a little wood-charcoal, so that the feces from the ester-feeding days were sharply marked off.

EXPERIMENT I The cat, after a preliminary starvation period of two days, was fed 50 grams hashed lean meat, 3.5 grams isomannid esters and 3 grams bone ash. The resulting feces were dried, broken up and extracted three to four hours in a Soxhlet extractor. The ether extract after filtering was evaporated to 50 cc and readings taken with the polariscope using a 1 dm tube.

Average of readings = $+0.05^\circ$

Subtracting reading of blank $+0.05^\circ$ —indicates complete absorption

EXPERIMENT II The same animal was fed 50 grams hashed lean meat, 4 grams of the isomannid esters and 3 grams bone ash The feces were collected and extracted as usual

Reading of the extract in 50 cc ether in 1 dm tube = $+0.29^{\circ}$

Blank = $+0.05^{\circ}$

Corrected reading = $+0.24^{\circ}$

Corresponding to an ester content of $+0.14$ gram

Absorption = 97.15 per cent

EXPERIMENT III The same animal, after the usual preliminary feeding with meat and charcoal was fed 50 grams of hashed lean meat, 4 grams of isomannid esters and 3 grams of bone ash The feces were collected and extracted as usual

Feces extract in 50 cc ether in 1 dm tube = $+0.39^{\circ}$

Blank = $+0.05^{\circ}$

Corrected reading = $+0.34^{\circ}$

Corresponding to 0.19 gram of ester

Absorption = 95.3 per cent

The results of the experiments indicate a practically complete utilization of the isomannid esters of the fatty acids used The slightly better absorption in Experiment I may have been due to the previous starvation In the paper immediately following this one are reported experiments on dogs, in the course of which, after feeding isomannid esters, the contents of the whole intestinal tract were removed and examined for unabsorbed esters The results bear out the findings of the above experiments, and show clearly that mannite esters of the fatty acids, if of suitable melting point, are as well utilized by the animal organism as are ordinary fats

Because of the excellent utilization of the isomannid esters of the fatty acid mixture used above (mainly lauric acid) it became of interest for purposes of comparison to determine the degree of utilization of the homologous isomannid ester of stearic acid⁴

The same animal was used as in the other experiments (a cat weighing about 1.6 kilo)

EXPERIMENT I After two days' starvation, the cat was fed 50 grams lean beef, 3 grams bone ash, and 4 grams isomannid distearate dissolved in 12 cc of cotton seed oil (The resulting mixture melted at $45^{\circ}\text{C}^{\circ}$, but remained soft at body temperature An unsuccessful attempt was made to obtain and feed a mixture melting at body temperature by using more cotton seed

⁴ Isomannid distearate is described in the second paper of this series
Bloor *loc cit*

oil The use of so much oil caused a diarrhoea) The feces were collected and extracted with ether as in the previous experiments

First passage of feces Ether extract in 50 cc ether in 1 dm tube reading 0.84°

Blank (for whole day), 0.05°

Corrected reading, 0.79°

Corresponding to a weight of ester of 0.43 gram

Second passage of feces Ether extract in 50 cc ether in 1 dm tube reading 1.10

Corresponding to a weight of ester of 0.60 gram

Total ester recovered in feces, 1.03 grams

Per cent absorption, 73 per cent

EXPERIMENT II The same animal was fed 3.5 grams of isomannid distearate in 10 cc of cotton oil with 50 grams lean beef and 3 grams bone ash. Feces collected in two portions and extracted with ether as usual

Portion 1 Reading of 50 cc ether extract in 1 dm tube = $+0.87^{\circ}$

Blank (for whole day) = $+0.05^{\circ}$

Corrected reading $+0.82^{\circ}$

Corresponding to 0.44 gram ester

Portion 2 Reading of 50 cc ether extract in 1 dm tube = $+1.03^{\circ}$

Corresponding to 0.55 gram of ester

Total ester recovered in feces = 0.99 gram

Per cent absorption = 72 per cent

The utilization of isomannid distearate by the animal body is quite comparable to that of a high melting fat, *e g*, tristearin⁵

SUMMARY

Mannitan and isomannid di-esters of lauric acid have been prepared and described

The isomannid esters of lauric and closely related fatty acids have been shown to be as well utilized by the animal organism as ordinary fats. The work is now being extended to the preparation of the esters of the higher fatty acids with the true carbohydrates

⁵ Arnschink *Zeitschr f Biol*, xxvi, p 434, 1890

ON FAT ABSORPTION

By W R BLOOR

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(Received for publication, March 27, 1912)

It is pretty generally believed that under normal intestinal conditions, most, if not all, of the food-fat is saponified in the intestine before absorption, and is absorbed as soaps. The question whether all fat *must* be split before absorption is still in doubt.

It has been shown that fat-like substances such as the petroleum hydrocarbons¹ and wool fat,² although they emulsify well, are not absorbed (a fact which in regard to the petroleum hydrocarbons has recently been disputed)³. Frank has demonstrated that the ethyl esters of the fatty acids, all of which form good emulsions and are readily saponified by the intestinal lipases, do not pass into the chyle unsplit.⁴ On the other hand there is much evidence to show that food fats may be transferred to the fat depots of the animal body without apparent change.

The research on the carbohydrate esters of the higher fatty acids⁵ was undertaken primarily in the hope of obtaining a fatty substance of such characteristic properties that it could be traced through the processes of absorption. With such a substance it was intended, among other things, to test further whether a fatty substance could pass into the chyle unchanged. The isomannid dilaurate described in the preceding paper seemed suitable for this purpose since it has great optical activity ($[\alpha]_D^{20} = +125^\circ$) which is lost on saponification, it emulsifies and saponifies readily, and

¹ Henriques and Hansen *Zentralbl f Physiol*, xv, p 313, 1900

² Connstein *Arch f (Anat u) Physiol*, p 30, 1899

³ Bradley *Proceedings Amer Soc Biol Chem*, Baltimore, Dec, 1911

⁴ Frank *Zeitschr f Biol*, xxxvi, p 568, 1898

⁵ Bloor *This Journal*, vii, p 427, xi, p 141

has been shown to be well utilized by the animal organism. Experiments were accordingly undertaken to determine whether isomannid dilaurate, when submitted to the processes of digestion and absorption could be detected in the chyle.

After a preliminary period of starvation the animals (dogs) were fed considerable amounts of the isomannid esters, described in the paper immediately preceding this, together with hashed lean beef, and after a sufficient time had elapsed for digestion to be well under way they were etherized, a cannula tied into the thoracic duct⁶ and the chyle collected for a period of three or four hours. The animals were then killed and the contents of the gastro-intestinal canal removed and examined for unabsorbed esters. The fat of the chyle was extracted by shaking out several times with ether, and finally the chyle was evaporated to dryness and again extracted with ether. The ethereal extracts were examined for optical activity. No attempt was made to determine the exact nature of the fat.

EXPERIMENT I A young dog (female), weighing 9 kilos, after starving two days, was fed 160 grams of hashed lean beef and 19 grams of isomannid esters of the fatty acids (mixture of lauric, myristic, etc.) obtained as described in the preceding paper. The ester mixture had a melting point of 25° and a specific rotation of $+88.4^{\circ}$. Time of feeding, 8.45 a.m. At 1.30 p.m. the animal was etherized and at 2.05 p.m. the cannula was securely tied into the duct—five hours and twenty minutes after feeding.

Collection of chyle

Portion 1 From 2.05 p.m. to 3.05 p.m., 33 cc. of very white milky chyle

Portion 2 From 3.05 p.m. to 4.05 p.m., 34 cc. chyle rapidly losing its white color

Portion 3 From 4.05 p.m. to 5.05 p.m., 35 cc. chyle of yellowish color

Portion 4 From 5.05 p.m. to 5.35 p.m., 14 cc. chyle of yellowish color
Animal killed

The blood pressure remained at a good height (130 to 140 mm.) throughout

Ether extract of chyle

Portion 1, acidified with sulphuric acid and extracted four times with warm ether. Extract = 0.8 gram. Per cent of fat in the chyle = 2.4 per cent. Optical activity, 0.8 gram in 35 cc. ether in 2 dm. tube, none

⁶ The operations were performed by Dr. D. E. Jackson of the department of Pharmacology of this school, to whom I take this opportunity of expressing my thanks.

Portions 2, 3 and 4 combined and extracted similarly Weight of total extract, 0.8 gram Per cent of fat in chyle of last three portions = 0.97 per cent Optical activity, none

The fat was saved to be united with material from later experiments for further examination

Examination of intestinal tract

The whole gastro-intestinal tract was removed shortly after death, slit throughout its whole length, and the contents scraped out under alcohol The stomach was found to be very much distended with gas and contained considerable undigested material, the small intestine was empty except for masses of intestinal worms The large intestine contained old fecal material only

The alcoholic mixture was allowed to settle in the cold, the clear liquid poured off and evaporated to dryness The alcohol-insoluble portion was dried separately After drying, the two portions were united and extracted several times with hot ether The extracts were united, washed with water, treated with a little bone black, filtered and evaporated to dryness The dry residue was taken up with ether and the ether extract examined for optical activity

Ninety-nine cubic centimeters of ether extract in 100 mm tube gave a rotation of $+1.88$, corresponding to a weight of ester of 1.9 gram

The ether extract was titrated with alcoholic alkali and phenolphthalein Acidity = 6.7 cc $\frac{N}{2}$ alkali corresponding to 1.34 gram lauric acid

The extract was now acidified, washed with water, evaporated to dryness, taken up with ether, filtered, again evaporated to dryness, and the residue weighed Weight, 4.0 grams

The ether-soluble material from the intestinal canal may then be listed as follows

	Grams
Undigested mannid esters	1.9
Fatty acids as lauric acid	1.34
Unidentified	0.76
	<hr/>
Total	4.0

Eight hours and fifty minutes (four hours under ether) after feeding, 90 per cent of the ester had been split and 83 per cent absorbed

EXPERIMENT II Dog (female), weight, 6 kilos, after two days starvation was fed 150 grams hashed lean meat and 13.2 grams of the isomannid esters This sample of ester melted at 20°C and had a specific rotation of $+100^{\circ}$ Time of feeding, 11.10 a m It was decided to begin the operation earlier than in the first experiment since it was found that digestion was almost complete at the time of the operation (five to six hours after feeding) The animal therefore, was etherized at 1.20 p m The cannula was in the duct and collection of chyle begun at 1.50 p m—two hours and forty minutes

after feeding The chyle was received in a small amount of ammonium oxalate solution, in order to obviate the difficulty of working with clotted lymph

Collection of chyle

Portion 1	1 50 to 2 50, 25 cc	white and milky
Portion 2	2 50 to 3 50, 35 cc	losing its dead white color
Portion 3	3 50 to 4 50, 28 cc	yellowish
Portion 4	4 50 to 5 20, 11 cc	almost clear Animal killed

The respiration was labored throughout, and the blood pressure was low and irregular, so that the animal required constant watching

Ether extractions of chyle

Portion 1 Weight of extract, 0 5 gram Fat content of chyle, 2 per cent
Optical activity, 0 5 gram in 20 cc ether in 200 mm tube, none (0 5 gram of the isomannid ester would have given a rotation of $+5.0^\circ$)

Portion 2 Weight of extract, 0 4 gram Fat content of chyle, 1 2 percent
Optical activity, none

Portions 3 and 4 Weight of extract, 0 1 gram

The extracted lymph from 1, 2, 3 and 4 was neutralized and evaporated to dryness with the aid of alcohol, and the dry material again extracted
Weight of extracted substance, 0 4 gram The chyle fat was united with that of Experiment I and saved for further examination

Examination of gastro-intestinal contents

The stomach and intestinal tract were treated as in Experiment I The stomach was very much distended with gas, and contained a considerable amount of undigested food, while the small intestine contained only mucus
The whole tract was scraped out and the contents dried and extracted with ether, as in the first experiment The ether extract was washed with water, treated with bone black, filtered and examined for optical activity

Volume of ether extract	475 cc
Rotation in 1 dm tube	$+1.0^\circ$
Corresponding to a weight of ester of	4 75 grams

Acidity The ether extract was evaporated to dryness, the residue taken up with chloroform and titrated with alcoholic alkali and phenolphthalein

Titration 11 84 cc $\frac{N}{2}$ alkali = 2 36 grams calculated as lauric acid, corresponding to 2 5 grams of the dilauric ester

The chloroform solution was acidified, washed with water and evaporated to dryness Weight of dry residue, 10 0 grams The constituents of the ether soluble portion of the intestinal contents were then

	<i>Grams</i>
Undigested ester	4 75
Fatty acid as lauric acid	2 36
Unidentified	2 89
	<hr/>
Total	10 00
Unabsorbed ester and fatty acid	7 1

Six hours and ten minutes (four hours under ether) after feeding, 64 per cent of the total ester had been split and 46 per cent absorbed

The chyle fat

The combined ether extract of the chyle from the two experiments was now examined. It was purified by solution in ether, evaporation to dryness, re-solution, neutralization, treatment with bone black, and filtering. The clear, slightly yellowish filtrate was evaporated to small bulk and examined for optical activity.

Twenty-five cubic centimeters of ether extract, containing 1.8 gram of the purified fat was examined in a polariscope using a 100 mm tube. Reading 0.08° , showing clearly that no unsplit ester had passed into the chyle (1.8 gram of the ester in 25 cc of ether would have shown a rotation of $+7.2^\circ$).

Melting point, 32°C

Refraction, Abbe refractometer— $n_D^{46} = 1.456$ (Trilaurin $n_D^{46} = 1.440$)

The fat was now saponified and the fatty acids separated. Melting point of mixed acids, 30°C

Mean molecular weight The mean molecular weight of the fatty acids was determined by titration.

0.357 gram in 100 cc chloroform with phenolphthalein = 1.69 cc $\frac{1}{2}$ alkali, from which the mean molecular weight was calculated to be 211.

Iodine number (Hübl method) 0.26 gram of fatty acid absorbed 0.043 gram I.

Iodine number, 16.5

The chyle fat therefore consists probably of trilaurin containing some triolein.

SUMMARY OF THE EXPERIMENTS

I Weight of dog, 9 kilos Time of operation, five hours and twenty minutes after feeding Duration of experiment, four hours
Weight of esters fed, 19 grams Weight of esters absorbed, 15.8 grams Esters in chyle, none

II Weight of dog, 6 kilos Time of operation, two hours and forty minutes after feeding Duration of operation, four hours and thirty minutes Weight of esters fed, 13.2 grams Weight of esters absorbed, 6 grams Esters in chyle, none

CONCLUSIONS

The results of the experiments show quite conclusively that none of the isomannid esters had passed unchanged into the chyle, although considerable quantities had been digested and absorbed. This result is in entire agreement with the findings of Frank⁷ with the ethyl esters of the fatty acids and emphasizes the probability that readily saponifiable fatty acid esters do not escape saponification under the favorable conditions in the normal intestine (excess of lipase, rapid removal of the products). Whether fatty substances of *any* kind may pass into the chyle unchanged remains to be proven.

⁷ Frank *Loc cit*

ECHINOCHROME, A RED SUBSTANCE IN SEA URCHINS

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INTRODUCTION

My interest in echinochrome arose from studies in permeability. In the same way that haemolytic agents cause haemoglobin to leave the red blood corpuscles, so do cytolytic agents cause echinochrome to leave the cells containing it. R. Lillie is of the opinion that this is due to the action of the cytolytic agent in increasing the permeability of the cell surface.

In the elaeocytes, wandering cells of the body fluid of *Arbacia punctulata*, the cytoplasm is crowded with spherical chromatophores. Some of these may be colorless, but usually they are colored bright red with echinochrome. Similar chromatophores, though not so close together, occur in the eggs. In the unfertilized egg they are evenly distributed throughout the cytoplasm. But after fertilization, the chromatophores all migrate to the surface within half an hour. During cleavage of the egg, they are massed in the cleavage furrows. The pigment occurs also in the test of this sea urchin, and gives the animal the characteristic color, which varies from a bright red (especially in young individuals) to a dark red, and may be almost black in old specimens.

In reference to the fact that the pigment may be caused to leave the chromatophores and pass into the cytoplasm and thence into the medium, the following questions may be asked: (1) How is the pigment held in the chromatophores? (2) What is its function? (3) What is its chemical nature? The present paper is concerned with these questions.

HISTORICAL

Echinochrome was studied spectroscopically by McMunn,¹ who found it in the elaeocytes of the sea urchins, *Strongylocentrotus lividus*, *Amphidotus cordatus*, *Echinus esculentus*? and *E sphaera*. The spectrum showed faint absorption bands, which varied with different solvents and different reactions of the same solvent. McMunn thought that he noticed changes in the spectrum on the addition of powerful reducing agents, such as stannous chloride, and concluded that echinochrome functioned as an oxygen carrier. However, the absorption bands in its spectrum are difficult to make out except in absolute alcohol (or glycerine) and in this solvent I observed that stannous chloride caused a precipitation of the pigment, which interfered with the examination.

A. B. Griffiths² attempted an elementary analysis of the substance. He dried the elaeocytes and extracted them with chloroform, benzol or carbon bisulphide. On evaporation of the solvent he analyzed the substance without further purification, although evidently it contained many impurities. From four analyses, he deduced the formula $C_{102}H_{99}N_{12}FeS O_1$, which would make C = 67.8 per cent, H = 5.5 per cent, and N = 9.3 per cent. He states that on boiling with mineral acids it is transformed into hæmato porphyrin, hæmochromogen and sulphuric acid ($E + \text{acid} = 2C_{34}H_{34}N_4O_4 + C_{34}H_{37}NFeO_6 + H_2SO_4$). Griffiths agrees with McMunn that echinochrome is an oxygen carrier, and states that the oxygen is held rather firmly, and in nature is removed only by the reducing action of the cell containing the pigment.

EXPERIMENTAL

The pigment in the elaeocytes, eggs and tests of *Arbacia*, shows no absorption bands, but after extraction it shows very similar bands in its spectrum to those described for echinochrome by McMunn. He published drawings of the spectra and measured the wave lengths corresponding to the edges of the bands. It is well known that bands become broader as the solution is more concentrated, and for that reason I measured the wave length of a line of the spectrum corresponding as nearly as could be determined to the center of each band. By taking the mean between the wave lengths of the edges of the band in McMunn's data I have compared his with mine. The discrepancies may be accounted for,

¹ McMunn *Quart Journ Micro Sci* (2), LV, p 469, 1885, LV, p 51, 1889.

² Griffiths *Compt rend soc biol*, CV, p 419, 1892, *Proc Roy Soc Edinburgh*, LV, p 117, 1892, *Physiology of the Invertebrata*, New York, 1892, *Respiratory Proteids*, London, 1897.

first by the fact that the mean is not the exact center of the band in a prism spectrum, and secondly there is a personal equation in observation. I found the pigment extracted from elaeocytes, eggs or tests to give about the same spectra, though a few isolated observations seemed to vary. These might have been due to decomposition products with different spectra.

	ETHER	ABSOLUTE ALCOHOL								H-O			
		Neutral			+HCl		+NH ₃			+HCl		+NH ₃	
My data	5296	4844	5504	5302	4844	5296	4844	5154	4844	5296	4844	5154	4844
McMunn			5512	5128	4845	5370	4998	5205	4848				

Neither McMunn nor Griffiths succeeded in crystallizing echinochrome. Dr A P Mathews had observed that on the addition of iodine in potassium iodide (KI₃) crystals form easily. In 1910 I obtained quantities of these crystals, but did not succeed in recrystallizing them without great loss by the formation of amorphous masses. The iodine compound in absolute alcohol showed an additional, but very dim band in the spectrum (wave length 5628 or 5696). It crystallized in red or orange needle-like crystals, triangular in cross section, sometimes rhombic in side view and often forming rosettes. They were but slightly soluble in water unless hot or containing acid, soluble in absolute alcohol (the rhombic crystals seeming more soluble than the needles) and slightly soluble in ether. If a solution in water is shaken with ether the latter is not colored. If an alkali is added to the KI₃ solution no crystals are formed (due to combination of the base with the echinochrome) but HCl does not prevent their formation.

Some of this iodine compound which was kept for several months in a dry state became more soluble in ether and crystallized in flat thin, red or orange rhombic plates. Perhaps the substance had decomposed with the liberation of iodine, for I succeeded in crystallizing the mother substance and obtained the same plates, in addition to red or orange needles, never triangular in cross section, but sometimes forming rosettes.

I extracted echinochrome from the tests with strong, slightly acidulated alcohol and purified it by repeated precipitation with

alkali and solution in acid alcohol, and filtration³ Finally I dissolved the precipitate in water plus HCl, filtered and shook the solution with ether The ether did not remove all of the echinochrome and the formation of haptogen membranes caused much loss of material The ether was evaporated at room temperature, as heat seemed to decompose the substance Occasionally a few crystals formed at the edges of the solution but the main mass of the residue was amorphous

The next season (1911) I tried to purify echinochrome without the use of acids or alkalies The body fluid of the sea urchins was allowed to clot and the elaeocytes thus obtained were placed directly into acetone, which extracted the pigment The extract was filtered and evaporated at room temperature The residue was washed with carbon tetrachloride (which does not easily dissolve echinochrome) to remove fats, and again dissolved in the smallest quantity of acetone and filtered to free it from traces of lecithin This solution was evaporated, dissolved in absolute ether and filtered to remove salts, evaporated to constant weight and analyzed by Dennstedt's method A mean of two analyses gave C = 51 per cent, H = 7.7 per cent The echinochrome purified by precipitating with alkali gave C = 53.3 per cent, H = 4.4 per cent, N = 1.5 per cent The nitrogen was determined by Kjeldahl's method and therefore may not be reliable, since the constitution of the molecule is unknown Traces of sulphur and phosphorus, possibly due to impurities were found, but no iron The ether-soluble crystals from spontaneous decomposition of the iodine compound gave C = 57.9 per cent, H = 6.5 per cent

It was stated above that echinochrome is precipitated by alkalies in alcohol I precipitated echinochrome with NaOH in 95 per cent alcohol and washed in the same alcohol to remove the excess of NaOH From the amount of NaOH that was neutralized by the pigment I concluded that it combined with from 18 to 25 per cent of Na Analysis gave C = 31.5 per cent, H = 6 per cent, Na = 19.5 per cent Therefore we may say that the echinochrome behaves as an acid, or else is amphoteric The former view is

³ Alkali does not precipitate it in water, the particular base was immaterial, ammonia was added but the presence of sea salts allowed the liberation of other bases

supported by the fact that on passing an electric current through the aqueous (colloidal) solution, the echinochrome shows a negative charge (is anodic) and again, if histological sections are placed in such a solution the acidophile portions are stained more strongly than the remainder. In fact its behavior is very similar to that of a weak solution of eosin, except that it is very easily washed out by alcohol.

However the substance is probably amphoteric (the acid character being stronger than the basic) since its aqueous solution is precipitated by phosphomolybdic and phosphotungstic acids but not by tannin.

From the analyses given above it would seem that no one has succeeded in obtaining echinochrome in a reasonably pure state. It is very unstable and probably breaks up into a host of decomposition products all having practically the same spectrum. If it is kept in the dry state for a great length of time, or is evaporated on a bath not over 50° for a shorter time, part of it becomes insoluble in ether but not in alcohol.

When heated in the combustion tube it first melts, then boils and sublimes as crystals on the top of the tube, then very soon turns brown and chars. After being crystallized from a solution in ether the crystals often become smaller and irregular in outline. Perhaps the crystals evaporate or lose water of crystallization, but I think that both these possibilities are improbable. The crystals may decompose into an amorphous substance.

On first obtaining crystals, I feared that they were crystals of some other substance merely colored by echinochrome, but this seems impossible from later observations.

In extractions made for the purpose of studying the lipoids in *Arbacia* eggs, red or brown substances (echinochrome or its decomposition products) appear in every fraction, rendering analysis difficult and indicating the instability and wide solubility of the substance.

In order to test the statement that it is an oxygen carrier I separated the cells from 50 cc of body fluid by the centrifuge, and mixed them with sea water to make 50 cc. This suspension, and 50 cc of sea water as a control, were placed in two similar graduated tubes. The air was pumped out for six hours (until the water boiled), air was then admitted and the tubes sealed. They

were shaken one-half hour and the volume of air measured at atmospheric pressure. The suspension had lost 1.25 cc, the control only 0.8 cc. In another experiment the suspension lost 0.95 cc and the sea water 0.8 cc. It was thought that in the absence of oxygen the cells would take the oxygen from the echinochrome. However no color change could be observed with the naked eye or the spectroscope, and the greater absorption of air by the suspension may have been entirely due to oxidation in the cells. In similar experiments, with an aqueous solution of the pigment, and distilled water for a control, and using pure oxygen, the two tubes gave the same absorption, as shown by two examples

Oxygen absorbed by H ₂ O	{ 1.0
	{ 1.1
Oxygen absorbed by echinochrome	{ 1.0
	{ 1.15

The question, how echinochrome is held in the chromatophores, cannot be fully answered. The chromatophores when free from pigment are highly refractive and stain strongly with the intravital stain, neutral red, and when fixed they stain strongly with Delafield's haematoxylin, indicating a lipid nature. The pigment may be in solution in the lipid.

The fact that the spectrum is different (shows no bands) in life from the spectrum of the extract may indicate chemical combination of the pigment with the chromatophores. The fact that echinochrome stains acidophile tissue may show a possible mode of such combination, if it be found that the chromatophores contain bases. However I do not think we can rely on the spectroscopic evidence, for the absorption bands are very faint in aqueous solution unless it be alkaline, and the cells containing the pigment interfere with the passage of light and make the observation difficult. I have never seen absorption bands in echinochrome extracted from the fresh cells with distilled water. The same statement is made by McMunn. If the substance is held by chemical combination why does it come out so easily?

The same argument may be made against the possibility that the echinochrome is held in the chromatophores because it is more soluble in them than in water. When the cell is stimulated mechanically or chemically the pigment comes out of the chromato-

phores with explosive rapidity. The cell need not be killed to accomplish this. The mere act of normal fertilization causes some of the chromatophores in the egg to lose their pigment.

The only alternative hypothesis I know of is, that the pigment is manufactured in the chromatophore, and cannot normally get out because the surface of this body is impermeable to it. An increase in permeability of the chromatophore allows the pigment to escape. Such an increase in permeability might be due to an aggregation change in the colloids of the limiting membrane or surface film.

Echinochrome is held in the chromatophores of the sea urchin's cells probably in the same way that chlorophyll is held in the chromatophores of the green plant cell.

THE PHYSIOLOGICAL ACTION OF SOME PYRIMIDINE COMPOUNDS OF THE BARBITURIC ACID SERIES

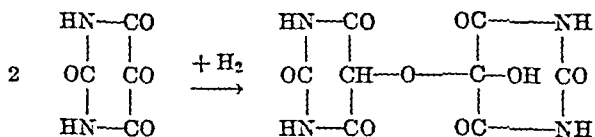
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(Received for publication, March 27, 1912)

Aside from the fact that certain pyrimidine compounds are constituents of the nucleic acid molecule, their possible biochemical importance is attested by a structural relation to the purines, creatine, creatinine, allantoin and other compounds of physiological interest. Moreover a few pyrimidines are known to have a marked pharmacological action.

The first substance of this type used in physiological experiments was alloxantine, which is formed by the reduction of alloxan.



Wöhler and Frerichs¹ fed 5 to 6 grams of this to men but could not recover any in the urine, nor was alloxan found. The urine was rich in urea, and a breaking down of alloxantine to urea and other products was believed to be probable. No mention is made of any toxic effects, although if any had been experienced they would undoubtedly have been described because the subjects were human beings.

Koehne fed alloxan and alloxantine in 8-gram doses to dogs. Each caused a mild diarrhea without other symptoms. No alloxan or alloxantine was excreted in the urine, but small amounts of oxalic and parabanic acids were found. Working independently of Koehne with the same compounds Lusini² obtained results different in some respects at least. In his experi-

¹ Wöhler and Frerichs *Ann d Chem u Pharm*, lxx, pp 335-349, 1848

² Koehne *Inaugural Dissertation*, Rostock, 1894, 39 pp

³ Lusini *Ann di chim e di farmacol*, xxi, pp 145-160, 1895, pp 41-257, and xxi, pp 341-351, 1895, pp 385-394 from *Chem Centralbl*, 1895, i, p 1074, ii, p 838

ments he found that both of these substances attacked the skin of frogs, dogs and rabbits. Both acted upon the cerebro-spinal centers, this action being divided into two periods, (a) hyper-reflex-excitability followed by rigidity, and (b) hypo-reflex-excitability and paralysis. This second stage had previously been noted by Curci,⁴ who because of the use of a larger dosage had overlooked the first stage. These and other minor effects varied slightly with the compound used, alloxan being in general more toxic than alloxantine. Alloxantine strongly reduced the hemoglobin of the blood both *in vitro*⁵ and *in vivo*. Among other phenomena produced in frogs by alloxan, mydriasis is noteworthy. Alloxan also had a powerful influence on the heart, the contractions were diminished in vigor, diastolic pauses lengthened, and finally the heart stopped in diastole.

According to Lusini, alloxan was non-toxic when given *per os*, 0.5 gram being easily borne. It did not reappear in the urine but parabanic acid and alloxantine were found. When Lusini fed alloxantine he recovered only slight traces in the urine. A small amount of dialuric acid was found together with parabanic acid and murexide in larger quantities. Lusini

reached the conclusion that the group, $\begin{array}{c} \text{HN} - \\ | \\ \text{OC} \\ | \\ \text{HN} - \end{array}$ is able to stimulate and then

inhibit the nerve centers, and that the grouping, $\begin{array}{c} \text{HN} - \text{CO} \\ | \\ \text{OC} \end{array}$ has no such power

It is, according to Lusini, the ketone-like CO which seems to have the stimulating property, and the abundance of these groups increases the toxicity of alloxan.

More recently Steudel⁶ has attempted to ascertain whether pyrimidines may be built up to purines in the animal body. The compounds used included those which Behrend and Roosen⁷ had described as intermediate products in the synthesis of uric acid in the chemical laboratory. At the outset it may be stated that a purine synthesis *in vivo* was not established. Steudel fed the substances to a bitch weighing 6.2 kg. in doses of 1 gram per day with meat and attempted to isolate them or their purine derivatives in the urine. 4-Methyluracil and 5-nitrouracil were found unchanged in the urine. 5-Nitrouracil-4-carboxylic acid, however, did not reappear in the urine. Steudel believes that it underwent a complete decomposition in the organism, although he does not consider the possibility of the non-absorption from the alimentary tract and does not report any analyses of

⁴ Curci. Cited by Lusini. *Ann di chim e di farmacol*, xxi, pp 145-160, 1895, from *Chem Centralbl*, 1895, i, p 1074.

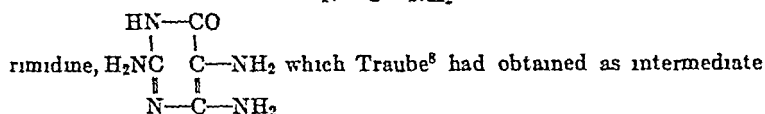
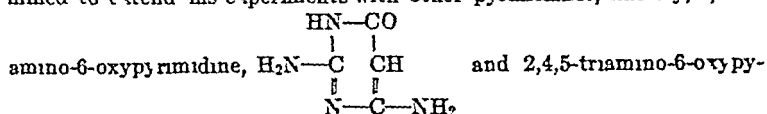
⁵ This property was described by Kowalewsky. *Centralbl f d med Wissensch*, xxv, pp 1-3, 17-18, 1887.

⁶ Steudel. *Zeitschr f physiol Chem*, xxxi, pp 285-290, 1901.

⁷ Behrend and Roosen. *Ann d Chem*, ccli, pp 235-256, 1889.

the feces Of the following pyrimidines none was recovered in the urine after feeding, nor was any difficultly soluble condensation product detected isobarbituric acid, isodialuric acid, thymine and uracil This author points out the striking difference in behavior between thymine (5-methyl-2,6-dioxypyrimidine) and 4-methyluracil Structurally they differ only in the position of the methyl group but the former is broken down in the body while the latter is not If, however, a nitro group is substituted for the methyl in thymine the physiological character of the pyrimidine is reversed, for it now passes unchanged through the kidney

Although a purine synthesis could not be demonstrated, Steudel determined to extend his experiments with other pyrimidines, namely, 2,4-di-



products in his synthesis of guanine Both were administered as the sulphates in 1-gram dose in the manner above described Both were reported to be toxic, which was surprising inasmuch as none of the other compounds had been accompanied by any untoward symptoms Feeding of the 2,4-diaminopyrimidine was followed by vomiting and the triamino compound provoked equally serious disturbances About an hour after the substance was taken, there occurred attempts at vomiting without any vomitus being ejected The animal had no appetite and lay on one side almost all day The urine contained protein, hyaline cylindroids and the unchanged triamino compound The last was recovered as the sulphate and identified by the violet color produced by saturating it with ammonia By subcutaneous injections the lethal dose for rats was determined as 0.2 gram for 2,4-diamino-6-oxypyrimidine sulphate and 0.1 gram for 2,4,5-triamino-6-oxypyrimidine sulphate Autopsy of the rats poisoned with the diamino substance revealed nothing characteristic, but the kidneys of the animals which had received the triamino compound contained numerous concretions and resembled microscopically the kidneys of dogs poisoned with adenine⁹

From these results, Steudel concluded that the attachment of amino groups to the pyrimidine ring transforms harmless, indifferent substances into poisonous ones The toxicity of adenine, 6-aminopurine, he regards as an analogous phenomenon in the purine series He believes that an examination of other amino derivatives of the pyrimidine and purine compounds will prove the universality of this law No analyses of the two amino-

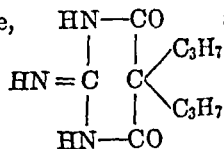
⁸ Traube *Ber d deutsch chem Gesellsch*, xxxiii, pp 1371-1383, 1900

⁹ Minkowski *Arch f exp Path u Pharm*, xli, pp 375-420, 1898

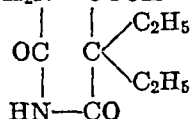
pyrimidines fed are presented by Steudel, nor are any data as to their solubility given

In a later contribution Steudel¹⁰ reported the investigation of other members of this series. Pseudo-uric acid and isouric acid did not result in a purine synthesis although both have been transformed into uric acid *in vitro*. A similar result was obtained when hydrouracil was fed. 2-Thio-4-methyluracil, like 4-methyluracil described above, was quickly excreted in the urine. 2-Amino-4-methyluracil, which differs from thiomethyluracil only in the substitution of an amino group for sulphur in the 2-position did not appear in the urine, nor was any other characteristic product found. Steudel concludes that none of the pyrimidines used by him are adapted to a synthesis of purine compounds in the dog.

The pharmacological action of some pyrimidines was studied by Fischer and von Mering¹¹ with interesting results. They discovered that certain alkyl derivatives possess an action similar to that of sulphonal. The latter, diethylsulphone-dimethylmethane, is rich in alkyl groups, and it was the idea of these authors to experiment with other alkyl organic compounds, many of which Fischer had synthesized, in the hope of ascertaining the essential or most effective groupings for hypnotic action. Of especial interest are the cyclic compounds employed, which are derivatives of barbituric acid and of malonyl guanidine. It was found that the 5-monoalkyl derivatives of barbituric acid have no hypnotic action, nor has the 5, 5-dimethyl derivative, but when both hydrogen atoms in the 5 position are replaced by alkyl groups, at least one being higher than methyl, the compound acquires sleep-producing powers. This reaches its maximum in 5, 5-dipropylbarbituric acid. Some of the compounds studied proved toxic, for example, substitution of the H in the 1 position by CH₃ or of the O in the 2 position by S transformed 5,5-diethylbarbituric acid into a toxic compound. However, 5,5-dipropylmalonyl guanidine,

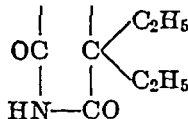


well as diethylmalonuric acid, $\text{H}_2\text{N} \quad \text{COOH}$ had no pharmacological



effect

5, 5-Diethylbarbituric acid, $\text{HN}-\text{CO}$ has been used widely in



¹⁰ Steudel *Zeitschr f physiol Chem*, LVIII, pp 136-142, 1903

¹¹ Fischer and von Mering *Therapie der Gegenwart*, N F, v, pp 97-101,

medicine as an hypnotic under the name "veronal," its sodium salt as "medinal," and the dipropyl compound to a less extent as "proponal." Fischer and von Mering¹² have found that most of the veronal is excreted from the body unchanged. Recently P. Fischer and Hoppe,¹³ Bachem,¹⁴ Gröber,¹⁵ and Jacoby¹⁶ have added many new facts to the literature of veronal.

Wolf¹⁷ injected uracil, thymine, and cytosine in 10 to 50 mgs doses into the circulation of cats, but observed no effect upon arterial pressure, intestinal volume, respiration, or rate of blood-clotting. Sweet and Levene¹⁸ fed thymine to a dog with an Eck's fistula (on the basis of Steudel's contention that thymine is destroyed by the normal dog). A marked diuresis resulted and thymine was found in the urine in considerable amount. This is interesting in view of the close relationship between this methylated pyrimidine and the methyl substituted xanthines theophylline, theobromine, and caffeine which are also diuretics.

Mendel and Myers¹⁹ have however recently shown that thymine is not completely destroyed normally by the dog, nor is uracil nor cytosine. Diuresis was not observed by them after the administration of thymine. The output of purines, creatinine and urea + ammonia was not influenced by administering any of these to rabbits, dogs or men. None of the compounds had any marked pharmacological effects. This is especially interesting because cytosine is an amino-pyrimidine, closely related to the compound alleged by Steudel to be toxic.

EXPERIMENTAL PART ²⁰

Dogs, rabbits, and guinea pigs were used in the physiological studies. The dogs were not catheterized, as the time relations were not of interest, but in the case of rabbits the urine was removed by artificial means in some experiments. The dogs' food always was mixed with bone so that the feces became firm and did not contaminate the urine. For the same reason the rabbits and guinea pigs were given some grain in addition to carrots. The compounds were administered subcutaneously, intraperitoneally, or by mouth.

¹² Fischer and von Mering *Therapie der Gegenwart*, April, 1904

¹³ P. Fischer and Hoppe *Münchener med Wochenschr*, 1909, p 1429

¹⁴ Bachem *Arch f exp Path u Pharm*, lxxii, p 228, 1910

¹⁵ Gröber *Biochem Zeitschr*, cxxi, p 1, 1911

¹⁶ Jacoby *Arch f exp Path u Pharm*, lxxvi, p 241, 1911

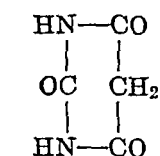
¹⁷ Wolf *Journ of Physiol*, cxxii, pp 171-174, 1905

¹⁸ Sweet and Levene *Journ of Exp Med*, ix, pp 229-239 1907

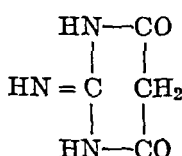
¹⁹ Mendel and Myers *Amer Journ of Physiol*, cxi, pp 77-105, 1910

²⁰ The experimental data in this paper are taken from the writer's dissertation for the degree of Doctor of Philosophy, Yale University, 1909

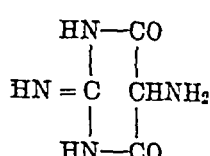
The substances used were barbituric acid and its amino-derivatives



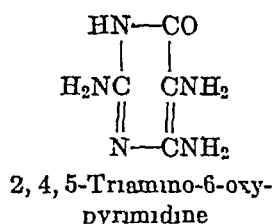
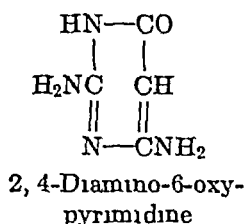
Barbituric acid
(Malonyl urea)



Malonyl guanidine



5-Aminomalonyl
guanidine



Barbituric Acid

Barbituric acid was made essentially according to Michael's²¹ method, the principle of which consists in condensing urea with diethylmalonate in the presence of sodium ethylate. The yield represented 71 per cent of the theoretical and the acid obtained gave the following results on analysis (Kjeldahl-Gunning method)

	Calculated for $\text{C}_4\text{H}_4\text{N}_2\text{O}_3$	Found
N	21.87	21.87
		21.65 ²²
		21.53 ²²

Barbituric acid crystallizes in two forms, the anhydrous as needles, and the hydrated as rhombic prisms. It is slightly soluble in water. A rough solubility determination showed that a 2.68 per cent solution can be prepared at 40 to 43°.

Efforts to obtain a method for estimating barbituric acid quantitatively in urine were unsuccessful, although a qualitative color test afforded a means of getting rough values colorimetrically. The difficulty lies in the fact that many of the properties of barbituric acid are possessed also by hippuric acid. As the latter

²¹ Michael *Journ f prakt Chem*, (2), xxxv, pp 449-459, 1887

²² These two analyses were made several months later

occurs constantly in all ordinary urines, and in considerable amount in the urine of herbivora, it proved an effective bar. Both compounds are precipitated by mercuric sulphate and by silver nitrate, both are soluble in ethyl acetate and amyl alcohol, and insoluble in ligroin, petroleum ether and benzene. The color reaction referred to is based on Baeyer's²³ observation that nitroso-barbituric acid, in the presence of ferrous acetate, yields a deep prussian blue color. The directions for this test are as follows: 3 cc of urine are treated with three drops of 2 per cent sodium nitrite solution, about 0.5 cc of 10 per cent sulphuric acid is added and the solution is now made alkaline with sodium carbonate solution, on addition of one or two drops of strong ferrous sulphate solution a beautiful blue appears in the presence of barbituric acid. When the expression " $\text{NaNO}_2\text{-FeSO}_4$ reaction" is employed hereafter it will be understood that this test is meant. Other members of this series give this reaction but thymine, cytosine and uracil do not.²⁴ Since urine frequently assumes a deeper color when subjected to this treatment, a direct colorimetric estimation was not attempted but a crude method was worked out, in which the greatest dilution allowing a positive test was considered the standard. It was thus found that a 0.0023 per cent solution of barbituric acid in water is the limit for this test, and hence the standard for comparison.

Barbituric acid is precipitated by mercuric sulphate solution. It gives Jaffé's reaction as applied to creatinine. A red color results when ferric chloride solution is added to barbituric acid.

The sodium salt was made by dissolving the acid in the amount of NaOH calculated to form the disodium salt, concentrating and allowing the salt to crystallize. Needle crystals were obtained, but that they were probably a mixture of the mono- and disodium salts is evident from the nitrogen determination.

²³ Baeyer *Ann d Chem u Pharm*, cxxvii, pp 199-236, 1863.

²⁴ None of the compounds of the barbituric acid series give the characteristic reactions of uracil, thymine or cytosine. For example, if thymine in substance be treated with diazobenzene sulphonic acid a reddish purple color results, tested in the same way barbituric acid gives a red, malonyl guanidine and cyanacetylguanidine a deep orange and the others a yellow or green color. When uracil or cytosine is dissolved in about 5 cc of water, bromine water added in slight excess and the solution boiled, a deep purple precipitate results on the addition of baryta water. None of the barbituric acid series studied gives this test.

N	Calculated for	Calculated for	Found
	$C_4H_2N_2O_2Na_2$	$C_4H_3N_2O_2Na$	
	16 28	18 66	17 58

As illustrations of the general method employed in the animal experiments two typical protocols will first be given

EXPERIMENT 1 A rabbit weighing 2 kg was given 0.519 gram barbituric acid in about 25 cc of water at 40°, hypodermically. The urines of the next two days were precipitated with mercuric sulphate, the precipitate decomposed with hydrogen sulphide and, after removing the mercuric sulphide, the colorimetric determination made. The amount excreted was estimated at 0.026 gram.

No hypnotic or toxic action was exerted by the compound. Its acidic character, however, made it harmful to the tissues at the point of injection, this caused an opening in the body wall which led to the death of the animal seven days after the injection.

EXPERIMENT 17 0.64 gram of sodium barbiturate in 45 cc water containing 0.1 cc $\frac{N}{16}$ NaOH at 38° were injected intraperitoneally into a rabbit weighing 1.6 kg. Diarrhea resulted in about two hours and this condition persisted for five days. The urines of the first two days gave positive $NaNO_2$ -FeSO₄ tests and these corresponded to 0.04 gram of barbituric acid.

These as well as other experiments with barbituric acid are tabulated on the opposite page.

From this table it is seen that the fatal termination of Experiments 1 and 3 must be ascribed to the acidic properties of barbituric acid, for when larger amounts of the sodium salt were given as in Experiments 12 and 17 no toxic effects resulted. The only physiological effect, which may be ascribed to its structure, is its diarrheal action, but a greater number of experiments need to be done to settle this point. In this connection it is interesting to recall the fact, noted above, that Koehne²⁵ observed a mild diarrhea after feeding alloxan and alloxantine. Again, the fact that barbituric acid has no hypnotic action harmonizes with Fischer and von Mering's²⁶ experiments on substituted barbituric acids, in which, as detailed above, they found that the lower the substituted alkyl groups, the less hypnotic the influence possessed by the complex. In barbituric acid, the lowest degree is reached and no hypnotic action is observed.

²⁵ Koehne Inaugural Dissertation, Rostock, 1894

²⁶ Fischer and von Mering *Therapie der Gegenwart*, v, pp 97-101, 1903

TABLE I
Animal Experiments Barbituric Acid (Malonyl urea)

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MODE OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per kilo-gram		
	kg	gram	gram		
(1) Rabbit	2 0	0 52	0 26	Subcutaneously	Not toxic except for necrosis at point of injection, which caused death seven days later Some excreted
(2) Rabbit	2 1	0 32	0 15	Subcutaneously	Not toxic Excreted about one-third (?)
(3) Rabbit	2 2	0 53	0 24	Intraperitoneally	Death in three days Diarrhea at first Diminished flow of urine (28 cc in two days) containing 0 09 gram (?) Autopsy revealed fibrinous adhesions in peritoneal cavity
(4) Rabbit	1 9	0 2	0 10	Intraperitoneally	Recrystallized preparation used Not toxic Under observation fifty-six days
(6) Rabbit	1 9	0 6	0 3	Per os	Marked diarrhea Excreted about $\frac{1}{2}$ in urine
(12) Guinea pig	0 5	0 3	0 6	Subcutaneously	Na salt used Not toxic Excreted 0 01 gram (?)
(17) Rabbit	1 6	0 64	0 4	Intraperitoneally	Na salt used Excreted about 0 04 gram (?) Diarrhea for five days otherwise not toxic Under observation thirty-one days

Malonyl Guanidine

In synthesizing malonyl guanidine Michael's²⁷ procedure was essentially followed. The pyrimidine was obtained in the form of its sodium salt which was dissolved in water and dilute NaOH, and the free pyrimidine precipitated with acetic acid. Malonyl guanidine crystallized in fine white needles which, after drying in a desiccator, were analyzed for nitrogen.

N	Calculated for		Found
	$C_6H_5N_3O_2 + H_2O$	$C_6H_5N_3O_2$	
	28.96	33.07	32.05
			31.91

The low nitrogen values are probably due to incomplete removal of the water of crystallization by simple desiccation. Inasmuch as the analysis was fairly close and the preparation was pure white no further purification was attempted. It was only slightly soluble in water. At 40 to 43° a 0.049 per cent solution was the strongest obtainable. This, of course, renders malonyl guanidine itself unsuitable for injection experiments and the sodium salt was accordingly used. In preparing this, the pyrimidine was dissolved in NaOH, as little in excess of the calculated amount as would bring about solution being used. On concentration, fine pale pink needles crystallized out. From the analyses, which follow, this salt must contain four molecules of water of crystallization which are lost in the desiccator very slowly.

N	Calculated for		Found (air dry)
	$C_6H_5NaN_3O_2 + 4H_2O$		
	19.00		18.77
N	Calculated for		Found (desiccated)
	$C_6H_5NaN_3O_2$		
	28.19		25.31

A method for recovering malonyl guanidine from urine is at once suggested by the slight solubility of the free substance. However, if urine is acidified and allowed to stand, uric acid and, if concentrated sufficiently, hippuric acid will also crystallize out. The $NaNO_2$ - $FeSO_4$ reaction described above for barbituric acid is also applicable to malonyl guanidine. The limit for this test in urine is 0.004 per cent. Another mode of estimation by means of this color reaction was tried as follows: 0.002 gram in

²⁷ Michael *Journ f prakt Chem*, *lix*, pp 26-43, 1894

3 cc water was converted to the prussian blue compound and dilutions made until the blue was no longer distinctly discernible in a 100 cc cylinder. The concentration just above this was considered the standard. By such a rough method it was found that a distinct blue can be seen when there is an amount corresponding to 0.0004 per cent present.

Sodium malonyl guanidine is not precipitated by ammoniacal silver nitrate solution, but is precipitated quantitatively by mercuric sulphate solution. With picric acid and alkali a red color is formed as in Jaffé's test for creatinine.

From the animal experiments (see Table II) it is seen that malonyl guanidine is non-toxic, at least in the doses for the animals used. The failure to detect the substance or a related compound in the urine of Experiment 5 may be due to the small amount injected.

TABLE II.

Animal Experiments Malonyl Guanidine

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MODE OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per Kilo-gram		
(5) Rabbit	2.2	0.09	0.04	Subcutaneously	Sodium salt used No effects. Not detected in urine.
(9) Rabbit	1.9	0.41	0.21	Subcutaneously	Sodium salt used No effects. Detected in urine.
(26) Rabbit	2.1	0.22	0.10	Subcutaneously	Sodium salt used Mild diarrhea, no other effects. All (?) excreted in urine.
(24) Dog	10	2.1	0.21	Per os	Free malonyl guanidine used. Some absorbed and excreted in urine. No toxic effects.

5-Aminomalonyl Guanidine

This compound is quite difficult to obtain in good yield as it decomposes very easily. The most advantageous method was found to be a modification of one described by Traube²⁸ in which the sulphate of this compound can be prepared directly from malonyl guanidine. The directions of Traube were followed as far as the formation of 5-aminomalonyl guanidine sulphate by reduction with H_2S , but instead of extracting this salt with hot water, the sulphur was removed by means of CS_2 and the sulphate converted into the hydrochloride by treatment with BaCl_2 . Traube's suggestion of adding alcohol to induce crystallization was not found to be advantageous since the crystals, when finally obtained, had a pink tinge. Consequently, the fluid was concentrated under diminished pressure and allowed to crystallize. Light yellow rosettes of needles formed very slowly.

Analysis of this preparation (A) by the Kjeldahl-Gunning method showed that, in spite of the tinge of yellow color, the salt was quite pure. Another preparation (B) made by the same method gave a higher nitrogen percentage.

	Calculated for (C ₄ H ₆ N ₄ O)HCl+H ₂ O		Found
N	28.57	{ A desiccated	28.55
		{ A desiccated	28.08
		{ B air-dry	29.41

Its solution, which is acid to litmus, very quickly turns red, owing undoubtedly to a slight oxidation. It stains the tissues red and has a faint disagreeable odor. Boiling with NH_4OH yields a solution colored like potassium permanganate and this changes to dark blue on addition of KOH . It will give the $\text{NaNO}_2\text{-FeSO}_4$ reaction, but not readily or brilliantly. The Jaffé color reaction for creatinine is not given by this salt. It is precipitated both by ammoniacal silver nitrate and mercuric sulphate, but as very little can be injected into an animal and as it was found to be toxic no attempt was made to isolate it from urine.

The toxicity of this compound is shown in the following illustrative protocols and the accompanying table (Table III) which summarizes all the experiments. The hydrochloride was used in each case.

²⁸ Traube *Ber d deutsch chem Gesellsch*, xxvi, pp 2551-2558, 1893

EXPERIMENT 7 November 30 3 10 p m A rabbit weighing 1 4 kg was given subcutaneously 0 37 gram in 35 cc water

3 20 p m Has defecated very soft stools Moves around restlessly

4 15 p m , 4 50 p m Apparently well

December 1 8 45 a m Rabbit found dead Autopsy kidneys are very light colored, intestines intensely reddened, liver, light brown, large amount of bloody fluid in peritoneal cavity

EXPERIMENT 23 March 11 11 00 a m A guinea pig weighing 450 grams was given 0 036 gram of the salt in about 10 cc water subcutaneously

March 12 2 15 p m Has eaten 60 grams carrots and 9 grams oats Urine, 64 cc , alkaline, specific gravity, 1 017, albumin present, but no casts

March 13 2 40 p m Has eaten 70 grams carrots and 3 grams oats Urine, 43 cc , alkaline, specific gravity, 1 024, large amount of albumin, granular, granular partly hyaline, and cellular casts found, $\text{NaNO}_2\text{-FeSO}_4$ test negative

March 14 2 35 p m Has eaten 85 grams carrots and 4 grams oats Urine 30 cc , alkaline, albumin present, casts

March 15 2 50 p m Has eaten 93 grams carrots and 3 grams oats Urine 57 cc , alkaline, specific gravity, 1 021, albumin, casts

March 16 8 40 a m Animal appears well Weight 390 grams The animal daily ate more food until March 20, when the usual amount (150 grams carrots and 15 grams oats) was entirely consumed On March 18, its weight had dropped to 360 gram but then rose to 440 grams on March 24 The urine still contained a trace of albumin On April 3—twenty-three days after the injection—the animal was still living and apparently well

EXPERIMENT 22 March 11 4 00 p m A female rabbit weighing 2 44 kg was given a subcutaneous injection of 0 19 gram in about 40 cc water

March 12 9 00 a m Stools partly diarrheal 2 15 p m No urine Has eaten 145 grams carrots but no oats

March 13 2 40 p m No urine Has eaten 130 grams carrots but no oats

March 14 11 00 a m No urine no feces Has eaten 85 grams carrots and 8 grams oats

March 15 2 50 p m Has eaten 46 grams carrots but no oats Urine, 163 cc , alkaline, specific gravity, 1 013, $\text{NaNO}_2\text{-FeSO}_4$ test negative, albumin and granular casts present, slight reduction of alkaline copper solution (after removing albumin)

March 16 8 40 a m Has eaten 35 grams carrots Animal is very weak, breathes slowly and can not hold its head up

10 05 a m Breathes more quickly but head is on floor of the cage

11 50 a m Still breathing, extremely weak

2 10 p m Found dead Urine, 52 cc , alkaline, specific gravity, 1 010 albumin and casts present, reduction positive

Autopsy Weight 2 26 kg All viscera hyperemic, blood of liver does not clot readily, kidneys edematous, bladder empty, animal is quite fat Sections of tissues preserved

TABLE III

Animal Experiments 5-Aminomalonyl guanidine

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MODE OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per kilo-gram		
(7) Rabbit	kg 1 4	gram 0 37	gram 0 26	Subcutaneously	Fatal in less than eighteen hours
(22) Rabbit	2 4	0 19	0 08	Subcutaneously	Albuminuria, casts, glycosuria Death in five days
(8) Rabbit	2 6	0 11	0 04	Subcutaneously	Fifty-three cubic centimeters urine in first forty-eight hours Albuminuria until fourth day No glycosuria Recovery
(10) Guinea pig	0 41	0 05	0 12	Subcutaneously	Albuminuria Death in four days Autopsy organs appear normal Blood does not clot readily
(27) Guinea pig	0 54	0 061	0 11	Subcutaneously	Not fed on day of injection Albuminuria mucus cylindroid seen Fatal in less than two days Autopsy one fetus present, large amount of bloody subcutaneous effusion Kidneys seem contracted
(25) Guinea pig	0 54	0 048	0 09	Subcutaneously	Albuminuria for at least seven days, casts and leucocytes in urine, recovery, under observation twelve days
(23) Guinea pig	0 45	0 036	0 08	Subcutaneously	Albuminuria, casts, recovery

TABLE III—Continued

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MODE OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per kilo-gram		
(20) Rabbit	kg 1 7	gram 0 18	gram 0 10	Per os	No symptoms, no albuminuria Under observation eighteen days
(21) Guinea pig	0 52	0 04	0 08	Per os	No symptoms, no albuminuria Under observation thirty days

From these results it appears that a lethal subcutaneous dose for rabbits is 0.08 gram per kg and for guinea pigs 0.11 gram per kg. It is also evident that when the compound is fed it is not toxic. In Experiment 20, the urine was repeatedly examined for substances giving the $\text{NaNO}_2\text{-FeSO}_4$ test but with negative results. The feces, however, in both Experiments 20 and 21 were tinged with pink at times. Probably not enough of the compound is absorbed from the alimentary tract at one time to prove toxic, it may be mentioned, however, that the hydrochloride is fairly soluble. That the compound acts mainly on the kidneys is evident from the protocols and the table, but substantiating evidence is given by the histological examination, made by Professor H. Gideon Wells to whom I am greatly indebted for the following report:

EXPERIMENT 22—Rabbit Kidney Shows extensive necrosis of the convoluted tubules, perhaps one-fourth of the tubules seen in section showing total necrosis of the epithelium. The necrotic epithelium desquamates into the lumen of the tubule which it fills up, and all stages of transition from masses of necrotic epithelium to granular and hyaline casts which pack the collecting tubules can readily be made out. These casts, being very abundant and staining intensely with eosin, give the sections a striking appearance. The tubular epithelium where not necrotic is strikingly little altered, some tendency to vacuolization of the cytoplasm being the chief abnormality noted. Glomerules congested, swollen, and in some a little granular material and occasional red corpuscles free in the space outside the tuft, in general the glomerules show relatively little change. There is an occasional small area of interstitial hemorrhage. To summarize, the poison has caused a marked necrosis of the epithelium of the convoluted

tubules, but without affecting other renal structures to any considerable degree

Liver No definite changes except the accumulation of masses of yellowish brown pigment in many of the stellate cells

Spleen Some of the endothelial cells of the splenic sinuses contain brownish pigment, otherwise no change The pigmentation of the liver and spleen suggests a hemolytic action by the poison

EXPERIMENT 27—*Guinea Pig Kidney* Shows the same necrosis of the secretory epithelium of the tubules and the same formation of casts as described in Rabbit 22, but very much less marked, only occasional tubules showing the lesion

Liver No pigmentation or other distinct changes

Spleen Much more pigment than in Rabbit 22 No other changes

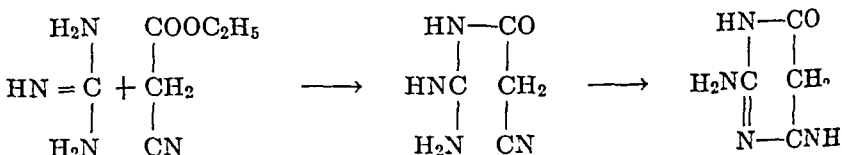
Adrenal No changes

EXPERIMENT 10—*Guinea Pig Kidney* Granular and hyaline casts are very abundant and conspicuous, although there are fewer tubules showing necrosis than in either of the other specimens When found it is typical, exactly the same in appearance as in 22 and 27 The casts much more often show desquamated epithelial cells within them Marked congestion, but no other changes The constancy of the finding of necrotic tubular epithelium in all three kidneys is conclusive evidence that this is a specific effect of the poison given

Liver No distinct alterations

2,4-Diamino-6-oxypyrimidine

Both 2,4-diamino-6-oxypyrimidine and its precursor, cyanacetylguanidine, were used in the experiments on animals They were made by Traube's²⁹ method with some modifications Guanidine hydrochloride, according to this procedure, is condensed with cyanethylacetate forming, in part, the pyrimidine, but mainly cyanacetylguanidine, which is easily converted into the pyrimidine by alkali



The yield of cyanacetylguanidine was 35.8 per cent of the theoretical, if this were the sole end-product The mother-liquor was of a dark red color and on concentration yielded a large amount

²⁹ Traube *Ber d deutsch chem Gesellsch*, LVIII, pp 1371-1383, 1900

of material which was used in the preparation of the pyrimidine. The first crop was recrystallized from hot water, pulverized and desiccated. To determine whether the substance obtained was cyanacetylguanidine or the pyrimidine, advantage was taken of the fact that the latter crystallizes with one molecule of water of crystallization while the former is water-free.

H_2O	Calculated for		Found
	$(C_4H_5N_3O) + H_2O$	$C_4H_5N_3O$	
	12.5	0.0	1.2

This preparation was consequently cyanacetylguanidine with very little, if any, pyrimidine admixture. Nitrogen determinations by the Kjeldahl-Gunning method gave low figures, perhaps because some HCN may have been formed and lost or because of a very slight admixture of the pyrimidine.

N	Calculated for		Found
	$C_4H_5N_3O$		
	44.44		41.99
			41.96

A much better yield is obtained by using guanidine sulphocyanide in place of the hydrochloride, as the mother liquor in this case is not as dark colored and may be evaporated to dryness without much loss of material. In this modification, when used as a step in the preparation of the pyrimidine, it is not necessary to remove the NaSCN formed until the 2,4-diamino-6-oxypyrimidine is precipitated as the sulphate, since the latter can be washed free from inorganic salts with water.

Cyanacetylguanidine is quite soluble in water—a 2.5 per cent solution being easily maintained at 40°—and is suitable for injection. Cyanacetylguanidine forms a rose red isonitroso compound (or is converted into the isonitroso derivative of 2,4-diamino-6-oxypyrimidine) on adding $NaNO_2$ and H_2SO_4 to its solution, as this is quite insoluble it may be isolated from the urine. According to Traube the isonitroso compound has an intense yellow or yellowish green color, however, with our preparation the brilliant red compound formed first and did not become yellow until additional acid was used. The color test with $NaNO_2$ and $FeSO_4$ as described above is also positive for cyanacetylguanidine.

For the transformation of cyanacetylguanidine into its isomer, 2,4-diamino-6-oxypyrimidine, it was put into boiling 2.5 per cent

NaOH, animal charcoal added, boiled a few minutes, and filtered into a beaker placed in an ice-bath. As some NH_3 is split off by boiling with alkali in this way, the operation must necessarily be performed quickly. The solution was now made weakly acid with H_2SO_4 and white or yellowish needle-like crystals of the sulphate of the pyrimidine appeared. When recrystallized from hot water large silky, grayish needles were obtained and these were again recrystallized from water in the presence of dilute H_2SO_4 and some charcoal, the crystals resulting were of a light yellow, almost white color.

According to Traube the sulphate, when recrystallized from water, contains one molecule of water of crystallization which is not driven off at 100° . Its composition is $(\text{C}_4\text{H}_6\text{N}_4\text{O}_2) \cdot \text{H}_2\text{SO}_4 + \text{H}_2\text{O}$. Our preparation agreed in its nitrogen content with this formula, as the following analyses indicate.

N	Calculated for $(\text{C}_4\text{H}_6\text{N}_4\text{O}_2) \cdot \text{H}_2\text{SO}_4 + \text{H}_2\text{O}$	Found
	30.43	30.42 30.27

This salt is sparingly soluble in water. A rough solubility determination showed that at 43° a greater concentration than 0.49 per cent could not be maintained and at a slightly lower temperature much of the substance instantly crystallized out. An aqueous solution gives a positive NaNO_2 - FeSO_4 test.

Because of the poor solubility no injection experiments were performed. However, Steudel's³⁰ experiment, in which he reports this compound toxic when fed to a dog, was repeated in exactly the same manner and with the same relative dosage.

EXPERIMENT 15. February 15. 10.20 a.m. Bitch weighing 9.6 kg. fed 180 grams chopped meat with bone meal, to which was added 1.55 grams of the sulphate of the pyrimidine.

10.20 to 11.40 a.m. Under observation almost continually. The animal, which has always been playful, shows no unusual behavior, but is apparently normal.

2.00 p.m. Animal still lively.

2.15 p.m. Ate some meat and drank water. No nausea observed.

5.10 to 5.20 p.m. Animal well.

February 16. 9.00 a.m. Fed meat, cracker and bone meal. Urine, 170 cc, specific gravity, 1.055, acid, no albumin. On adding NaNO_2 and H_2SO_4 .

³⁰ Steudel *Zeitschr. f. physiol. Chem.*, XLII, pp. 285-290, 1901.

a rose-colored precipitate appeared which was filtered off and washed with hot water, alcohol and ether. For the total volume of urine this would have amounted to 1.17 grams. It was dissolved in KOH, reprecipitated by HCl, filtered etc. and analyzed by the Kjeldahl-Gunning method (for nitrates)

N	Calculated for isonitroso derivative of		Found
	2,4-diamino-6-oxypyrimidine (=C ₄ H ₅ N ₃ O ₂)	monamino-di-oxypyrimidine (=C ₄ H ₄ N ₃ O ₂)	
	45.16	35.89	
			39.25
			39.56

Feeding suspensions of the salt to a guinea pig and to a rabbit gave similar non-toxic results (see Table IV). The sulphate, as in Steudel's investigation, was used in every case.

TABLE IV

Animal Experiments 2,4-Diamino-6-oxypyrimidine

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MANNER OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per kilo-gram		
(15) Dog	kg 9.6	gram 1.55	gram 0.16	Per os	No toxic effect. Large proportion excreted, deaminized (?)
(18) Guinea pig	0.16	0.14	0.87	Per os	Fed in saccharose suspension from pipette. No symptoms. Under observation seven days.
(19) Rabbit	1.96	0.51	0.26	Per os	Given in suspension in water. No albuminuria. NaNO ₂ - FeSO ₄ test positive. Unable to obtain an isonitroso compound as in Experiment 15.

It is therefore evident that this pyrimidine is *not toxic* when given *per os* as the sulphate. Doses larger than those reported toxic by Steudel were without effect upon the rabbit and guinea pig as Experiments 18 and 19 indicate.

2,4,5-Triamino-6-oxypyrimidine

This pyrimidine was prepared according to Traube's³¹ directions and was isolated as the sulphate. When crystallized quickly the salt appears as small rods or rectangular prisms but if allowed to crystallize slowly large needles are formed. After desiccation, an analysis gave the following results

N	Calculated for	Found
	(C ₄ H ₃ N ₅ O)H SO ₄ +H ₂ O	
	27 24	27 87

The solubility of this salt is about the same as that of the diamino compound, *re*, it was found to be possible to obtain a 0.49 per cent solution at 43°. In this case, however, the fluid became dark during the manipulation and, after drying, the residue was dark brown in color. It is thus evident that some chemical change—a decomposition or oxidation—occurred and hence the determination can only be regarded as an evidence of the very slight solubility of the substance at low temperatures and of its instability, when in solution, at a high temperature.

According to Traube, if an ammoniacal solution of the sulphate be shaken so as to afford contact with the air the fluid assumes an intense violet color resembling permanganate solution. This reaction, according to our experience, is better performed and with more uniform success, if a few milligrams of the substance are placed on a porcelain surface together with one or two drops of NH₄OH and evaporated to dryness on a water-bath, the violet tinge is here seen against the white surface. In trying to dissolve some of the salt in 50 per cent alcohol it was discovered that although very little went into solution the latter became colored with this same violet tint. Thus pyrimidine also resembles uric acid in two reactions, namely, the murexide and Schiff's tests, the murexide test is given very brilliantly indeed. Addition of bromine water to an aqueous solution was found to produce a deep reddish-purple color which vanished, leaving a yellow solution, when the bromine was in excess. The NaNO₂-FeSO₄ reaction is positive if the triamino pyrimidine be first dissolved in boiling water, this is probably due to a trace of the diamino being formed by the action of the water as, theoretically, if the 5 position is occupied by an amino group no isonitroso derivative can be formed.

³¹ Traube *Ber d deutsch chem Gesellsch*, **xxiii**, pp 1371-1383, 1900

In using this salt in physiological experiments we again obtained results quite different from those reported by Steudel

EXPERIMENT 14 February 11 2 45 p m Bitch weighing 9 8 kg (same animal as in Experiment 15) was given 1 58 grams of the sulphate of this pyrimidine mixed with about 180 grams of chopped meat and some bone meal No unusual symptoms were noticed by 4 00 p m

4.30, 5.00, 5.30, 7 40, 9 15 p m , animal observed and was well and playful

February 12 8 50 a m Animal well Urine, 134 cc , dark orange-red in color, specific gravity, 1 049, acid, no albumin Some of the urine was made acid with H SO_4 and was concentrated to small volume HgSO_4 solution was added and the precipitate filtered off, a few crystals were found and, as they gave a violet color on treatment with NH_4OH and evaporation, were probably some triamino-sulphate which had crystallized before adding the HgSO_4 The mercury precipitate was unfortunately lost through an accident

4 00 p m Fed meat and bone

February 13 a m Urine light yellow in color

Relatively larger doses were fed in suspension to a rabbit and a guinea pig with similarly negative results, these are summed up in the following table (Table V)

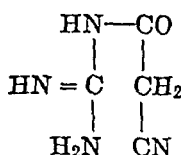
TABLE V

Animal Experiments 2, 4, 5-triamino-6-oxypyrimidine

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MANNER OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per kilo-gram		
	kg	gram	gram		
(14) Dog	9 8	1 58	0 16	Per os	No toxic effect Some excreted (?) Urine red
(13) Rabbit	2 5	0 2 0 5	0 08 0 20	Per os	No toxic effect Second dose four days after first Urine red after second dose
(10) Guinea pig (young)	0 13	0 13	1 0	Per os	Fed, suspended in saccharose solution, from a pipette No toxic effects Urine colored dark red

It is accordingly evident that even the triamino compound, which Steudel claims is the more toxic of the two, has no harmful influence upon the organism when administered by way of the mouth

Cyanacetylguanidine



The preparation and properties of cyanacetylguanidine are described above in the description of the process of making 2,4-diamino-6-oxypyrimidine

This compound was used because it is a precursor of the diamino and triamino pyrimidines just described and *might readily be present as an impurity if these compounds were carelessly prepared*. Inasmuch as from the following experiments it is seen to be toxic after injection, a reason for the difference between our results and Steudel's is thus suggested

EXPERIMENT 28 March 30 12 30 m Injected subcutaneously, into guinea pig weighing 680 grams, 0.38 gram cyanacetylguanidine in 15 cc water

4 00 p m Animal shows hyperexcitability

6 20 p m Still very excitable

March 31 1 15 p m Apparently well except for continued hyperexcitable state, which is not as great as on the previous day

4 00 p m Has eaten 15 grams oats and 95 grams carrots during twenty-four hours Weight 668 grams Urine, 43 cc, alkaline, specific gravity, 1.031, no albumin present, strong NaNO_2 - FeSO_4 test, upon addition of NaNO_2 in substance, and H_2SO_4 a pink isonitroso derivative was obtained which amounted to 0.161 gram, if computed to total volume This was analyzed with the following results

	Calculated for $\text{C}_4\text{H}_7\text{N}_5\text{O}_2$ (= isonitroso derivative of cyanacetylguanidine)	Found
N	45.16	36.47

5 10 p m Apparently well

April 1 4 00 p m Has eaten 15 grams oats and 105 grams carrots Weight, 664 grams Urine, 46 cc, alkaline, specific gravity, 1.030, no albumin, NaNO_2 - FeSO_4 test positive

April 2 4:00 p m Weight, 667 grams, Urine, 38 cc, $\text{NaNO}_2\text{-FeSO}_4$ test negative

EXPERIMENT 31 April 2 11 45 a m Young guinea pig, weighing 191 grams, given 0.4 gram cyanacetylguanidine in 17 cc water by subcutaneous injection

12 15 m Apparently well

2 10 p m Animal found in violent spasms, especially the posterior parts of the body There is hyperexcitability

2:20 p m Head raised a little more and pig runs around some, pawing at its chin at intervals Twitchings continue

4 13 p.m Violent convulsion, lies on its side and moves its limbs rapidly

4 18 p m Animal gradually rights itself and grips the side of the wire cage with its teeth Waves of convulsions, starting at the posterior part and running forward, occur

4:22 p m Dies in the same position, body quickly in rigor The urine excreted, 2 cc, was found to contain no albumin but on addition of NaNO_2 and H_2SO_4 a pink precipitate appeared which after dissolving in Na_2CO_3 and adding FeSO_4 produced the deep prussian blue color

EXPERIMENT 30 March 31 2:55 p m A dog weighing 5.8 kg was fed 100 grams chopped meat containing 0.94 gram cyanacetylguanidine

3 10 to 3:20, 4:25 to 4:30 p.m Apparently no effects

4 50 p.m Drank water, no nausea

April 1 9 15 a.m Dog apparently well 3:00 p m Fed meat, lard, bone and cracker meal Urine, 226 cc, acid, specific gravity, 1.025, no albumin, strong $\text{NaNO}_2\text{-FeSO}_4$ test To an aliquot portion was added solid NaNO_2 and H_2SO_4 and the reddish brown precipitate which amounted to 0.389 gram analyzed

Calculated for
 $\text{C}_4\text{H}_5\text{N}_5\text{O}_2$ (= isonitroso
 derivative of
 cyanacetylguanidine)

Found
 34.43

N

45.16

April 2 3:00 p m Dog well Urine, 138 cc, specific gravity, 1.032, acid, no albumin, strong $\text{NaNO}_2\text{-FeSO}_4$ test No loss of appetite or other unfavorable symptoms

April 3 9:30 a m Dog well Weight, 5.6 kg Urine gives uncertain $\text{NaNO}_2\text{-FeSO}_4$ test

These and one other experiment are summarized in Table VI. The low nitrogen values found in Experiments 28 and 30 suggest the possibility of a deamination of cyanacetylguanidine in the body. The substitution of O for NH in its isonitroso derivative would result in a compound containing 35.90 per cent of nitrogen, the figures found, 36.47 per cent and 34.43 per cent, correspond with this percentage.

TABLE VI

Animal Experiments Cyanacetylguanidine

NUMBER AND ANIMAL	WEIGHT	AMOUNT GIVEN		MANNER OF ADMINISTRATION	REMARKS AND RESULTS
		Total	Per kilo-gram		
	<i>kg</i>	<i>gram</i>	<i>gram</i>		
(28) Guinea pig	0 68	0 38	0 56	Subcutaneously	Hyperecitability Excreted considerable as a deaminized (?) substance
(31) Guinea pig	0 19	0 40	2 1	Subcutaneously	Hyperecitability Violent convulsions Fatal in four and three quarters hours
(29) Dog	8 4	0 70	0 08	Per os	No symptoms Urine gave positive Na-NO ₂ -FeSO ₄ test No albuminuria
(30) Dog	5 8	0 94	0 16	Per os	No harmful effect Considerable excreted as a deaminized (?) substance

This toxic action agrees with the results of some unpublished trials by Mr J J Costello, who observed similar effects in Professor Mendel's laboratory when the sulphate of this compound was subcutaneously injected A few of his figures follow

Dose per kilogram
of guinea pig

0 92

0 96

0 96

1 02

2 26

Results

Hyperecitability-recovery

Hyperecitability for two days-recovery

Death in sixteen hours

Death in fourteen hours

Death in three and one-half hours

DISCUSSION

In considering the physiological and pharmacological behavior of the members of this series the most striking fact is the toxicity of 5-aminomalonyl guanidine with its chief effect upon the epithelium of the convoluted tubules Its harmlessness when adminis-

tered *per os* may be due either to an absorption so slow as to allow of elimination before a toxic concentration is reached, or to a transformation—perhaps by deaminization—into a non-toxic compound in the intestinal wall. The toxicity after subcutaneous administration may possibly be attributable to some hydrolytic or oxidation product formed during solution inasmuch as the solution quickly assumes a red color.

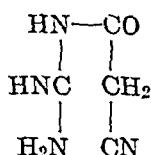
The absence of hypnotic powers in barbituric acid and malonylguanidine is in harmony with the ineffectiveness of the lower alkyl barbituric acid derivatives and of 5,5-dipropylmalonylguanidine²². The diarrheal action of barbituric acid is noteworthy because of a similar action ascribed to alloxan²³.

Steudel's²⁴ claim that 2,4-diamino-6-oxypyrimidine and 2,4,5-triamino-6-oxypyrimidine are toxic, cannot be substantiated. In duplicating his experiments in which he fed these compounds to a dog, no similar results could be obtained, the animal used was a very playful one as was Steudel's but it did not become less lively after ingesting these substances, nor was vomiting or albuminuria observed or any other of the effects noted by that author. The lethal doses for rats he gives as 0.2 gram and 0.1 gram for the sulphates of the diamino and triamino compounds, respectively, when injected subcutaneously. The smallest volumes which can possibly contain these amounts at 43° are 40 cc and 20 cc respectively. Moreover, it was shown above that such concentrations are not suitable for injection and this leads us to believe that Steudel used products which were more soluble than these aminopyrimidines. Moreover he published no analyses of his compounds. Cyanacetylguanidine, however, is a precursor of both pyrimidines, it is quite soluble as is also its sulphate, and finally, when injected subcutaneously it is toxic. These properties would indicate that this compound was an admixture of Steudel's preparations and would account for their toxic action. However, when fed to dogs, cyanacetylguanidine is not toxic although his preparations were, and the only apparent explanation for this is that still another contaminating substance was responsible in this case. That cyanacetylguanidine is toxic is not surprising since, from its structure,

²² Fischer and von Mering *Therapie der Gegenwart*, v, pp 97-101, 1903

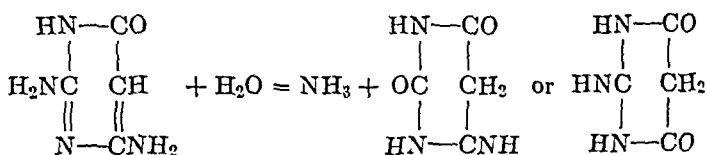
²³ Koehne *Inaugural Dissertation*, Rostock, 1894, 40 pp

²⁴ Steudel *Zeitschr f physiol Chem*, xxxi, pp 285-290, 1901

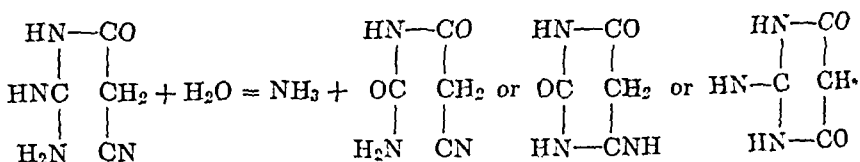


it might possess the properties of guanidine or of nitriles. Guanidine, the toxicity of which has long been known, causes³⁵ peculiar shaking movements of the head and ears, paralysis of the hind limbs, clonic muscular contractions and muscular twitchings of the entire body. Different nitriles have different effects but the typical phenomena are described³⁶ as vomiting, dyspnoea, tetanic convulsions and opisthotonus. Hence, probably cyanacetylguanidine embraces some of the toxic effects of both of these poisons (see Experiments 28 and 31).

The behavior of 2,4-diamino-6-oxypyrimidine and cyanacetylguanidine in the body affords suggestions for further work upon the intermediary metabolism of these substances, as the few experiments indicate that a deamination may occur *in vivo*. The differences between the theoretical percentage of N for the compounds administered and those recovered from the urine are too great (6 to 11 per cent) to be ascribed to the method of analysis or to faulty technique. Moreover, an analysis of the pure isonitroso derivative of the diamino pyrimidine by the same method gave a satisfactory nitrogen value. The possibilities for the transformation of this pyrimidine are shown by the following scheme



With cyanacetylguanidine a somewhat similar problem is presented as deamination can result in one of three compounds



³⁵ Gergens and Baumann *Arch f d ges Physiol*, vii, pp 205-214, 1876, Pommerenig *Beitr z chem Physiol u Path*, i, pp 561-566, 1901

³⁶ Kobert *Lehrbuch der Intoxikationen*, Stuttgart, ii, p 862, 1906

If either of the last two complexes result it is of great interest as no precisely similar transformation of an acyclic into a cyclic compound is known in physiology



Lusini's conclusion that the grouping OC has first a stimulat-



ing and then an inhibiting action on the nerve centers and that



the grouping $\begin{array}{c} | \\ \text{OC} \end{array}$ has no such power can not be substan-



tiated inasmuch as barbituric acid, which is non-toxic, contains this urea grouping and differs very little in structure from alloxan which Lusini found to be toxic

SUMMARY

The administration of barbituric acid *per os* is followed by no marked physiological effects except diarrhea, when given subcutaneously the free pyrimidine has a local action on the tissues due to its acid properties. The sodium salt has no local action.

Malonyl guanidine when fed, or when injected subcutaneously as the sodium salt, provokes no noteworthy symptoms. 5-Aminomalonylguanidine hydrochloride, 2,4-diamino-6-oxypyrimidinesulphate and 2,4,5-triamino-6-oxypyrimidine sulphate, when fed, are also without marked action.

Subcutaneous injection of 5-aminomalonylguanidine hydrochloride leads to grave changes in the tubular epithelium of the kidney, casts and albumin abound in the urine, and death frequently results.

2,4-Diamino-6-oxypyrimidine sulphate and 2,4,5-triamino-6-oxypyrimidine sulphate, which Steudel reported as toxic, are too insoluble to inject in appreciable quantity. Inasmuch as cyanacetylguanidine, a precursor of both of these, is quite soluble, and was found to be toxic when injected subcutaneously, doubt is expressed as to the purity of the diamino and triamino pyrimidines used by Steudel, especially as this author also observed nausea, etc., after feeding them to dogs, whereas no symptoms whatever occurred in the present investigation under similar conditions.

A color reaction is described which is common to all of this series, although 2,4,5-triamino-6-oxypyrimidine and 5-aminomalonylguanidine do not react well. By aid of this reaction and in other ways, evidence was gained that, after administration of a compound of this series there was excreted in the urine the compound used (or a derivative) in every case except with 5-aminomalonylguanidine, and perhaps 2,4,5-triamino-6-oxypyrimidine.

Evidence is presented to indicate that 2,4-diamino-6-oxypyrimidine and cyanacetylguanidine may be deaminized in the body.

My thanks are due Prof. Lafayette B. Mendel who directed the physiological investigations and Prof. Treat B. Johnson, who aided and advised in the syntheses of the compounds employed as well as in the questions of organic chemistry involved.

PHYTIN AND PHOSPHORIC ACID ESTERS OF INOSITE

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(Received for publication, April 2, 1912)

In continuation of the physiological investigation concerning the metabolism of the organic-phosphorus compound known as phytin, which has been and is being carried out at this institution by Dr. Jordan, a closer study of the chemical properties of this substance, phytin, became necessary. Much work has already been done and reported on this subject by various investigators. Definite information, however, concerning different kinds of salts formed by the free phytic acid or inosite phosphoric acid is seldom met with in the literature. Frequently impure salts have been analyzed.

Posternak, who first successfully prepared phytin in pure form,¹ also studied its chemical properties. Among the salts mentioned² is one, calcium-magnesium, as well as one crystalline, calcium-sodium, double salt, for which he gives the formula, $2C_2H_4P_2O_5Na_4 + C_2H_4P_2Ca_2 + 8H_2O$. Winterstein³ describes a calcium-magnesium compound which, after removing the calcium with oxalic acid and precipitating with alcohol, contained 42.24 per cent P_2O_5 and 12.97 per cent MgO . Patten and Hart,⁴ working in this laboratory, isolated from wheat bran an impure magnesium-calcium-potassium compound. Levene⁵ describes a semi-crystalline barium salt which corresponds to a tetra-barium phytate. Vorbrodt⁶ mentions a crystalline barium salt obtained by partially

¹ *Rev. gén. de bot.*, **xxi**, p. 5, *Compt. rend. acad. des sci.*, **cxxxvii**, p. 202.

² *Compt. rend. acad. des sci.*, **cxxxvii**, pp. 337 and 439.

³ *Ber. d. d. chem. Gesellsch.*, **xxx**, p. 2299.

⁴ *Amer. Chem. Journ.*, **xxxi**, p. 566.

⁵ *Biochem. Zeitschr.*, **xvi**, p. 399.

⁶ *Anzeiger Akad. Wiss. Krakau*, 1910, Series A, p. 414.

neutralizing phytic acid with barium hydroxide and evaporating in vacuum, to which he assigns the formula, $C_{12}H_{26}O_{46}Ba_7P_{11}$. Although crystalline, this compound was undoubtedly impure. By neutralizing the mother-liquor from the above with barium hydroxide he obtained an amorphous precipitate of the composition C, 5.75, H, 0.77, Ba, 52.97, P, 11.60 per cent. This corresponds approximately with a hexa-barium phytate.

Of the several salts mentioned in this paper some were obtained from commercial phytin and from an organic-phosphorus-magnesium compound by precipitating with barium chloride and barium hydroxide, others were prepared from previously purified phytic acid. These products will be more fully described in the experimental part.

The tri-barium phytate, $C_6H_{12}O_9[(PO_3H)_2Ba]_3$, is obtained pure as an amorphous white powder by repeatedly precipitating barium phytate in 0.5 per cent hydrochloric acid with a like volume of alcohol. It may also be obtained in crystalline form by dissolving the amorphous salt in a 10 per cent solution of phytic acid in which it is very soluble and from which it again slowly crystallizes out on standing at ordinary temperature.

A penta-barium phytate, $C_6H_{14}O_{27}P_6Ba_5$, is obtained when a solution of the tri-barium phytate in 0.5 per cent hydrochloric acid is neutralized with barium hydroxide and then made faintly acid with acetic acid.

The penta-barium ammonium phytate, $C_6H_{12}O_{27}P_6Ba_5(NH_4)_2$, is obtained when the above mentioned amorphous tri-barium salt is digested with dilute ammonia.

The penta-magnesium ammonium phytate, $C_6H_{12}O_{27}P_6Mg_5(NH_4)_2$, is thrown down as a white amorphous precipitate when excess of magnesia mixture is added to an aqueous solution of phytic acid, or when ammonium phytate is precipitated with magnesia mixture.

A tetra-cupric di-calcium phytate, $C_6H_{12}O_{27}P_6Cu_4Ca_2$, in nearly pure form is obtained when a slightly acid solution of calcium ammonium phytate is precipitated with excess of copper acetate. If the magnesium ammonium phytate is precipitated under the same conditions an impure compound is obtained which contains about 1 per cent Mg, 0.6 per cent N, 34 per cent Cu and 15.6 per cent P. No effort was made to obtain these salts pure. It was

only desired to find out to what extent other bases were removed when precipitating with copper acetate

Starkenstein⁷ claims that commercial phytin always contains free inosite together with inorganic phosphates and that merely drying the substance at 100°C causes nearly complete decomposition into inorganic phosphate and free inosite

That phytin is so easily decomposed seemed very improbable as several months' work on the substance has shown that it is relatively stable when pure and when no mineral acids are present. Moreover Contardi⁸ reports that when phytin is heated in an autoclave with pure water for several hours to a temperature of 200°C only very small quantities of inosite could be isolated

In order to determine if inosite is present in determinable quantity 100 grams of commercial phytin in the form of the acid calcium salt, imported from Europe and which had been kept in the laboratory for several years, was shaken up with 1 liter of water, filtered at once and washed with water. The filtrate was precipitated with barium hydroxide, again filtered and the excess of barium precipitated with carbon dioxide and the filtrate from the latter evaporated on the water-bath. In the very slight residue which remained, consisting mostly of barium carbonate with a trace of barium chloride, no trace of inosite could be detected by the most painstaking method of isolation. Of the same phytin, 100 grams were dried to constant weight at 115°C and was then treated in the same manner. Even here no trace of inosite could be obtained. Subjecting to the same treatment 50 grams of the same phytin, after previously mixing with 0.5 gram inosite, resulted in the recovery of 0.4 gram inosite.

This proves that phytin is by no means so easily split as Starkenstein claims. The results in his case may have been due to other causes besides mere drying at 100°C.

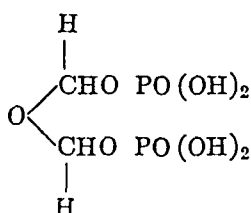
The same author (*loc cit*) also states that when phytic acid is precipitated with ammoniacal magnesia mixture it is not the magnesium ammonium compound which is formed but only the difficultly soluble magnesium phytate. This is an error. Under these conditions the previously mentioned penta-magnesium ammonium phytate, $C_6H_{12}O_{27}P_5Mg_5(NH_4)_2$, is formed.

For the free phytic acid Posternak⁹ proposed the empirical formula, $C_2H_8O_9P_2$, which he considered to have the following constitution

⁷ *Biochem Zeitschr* **xx**, p 59

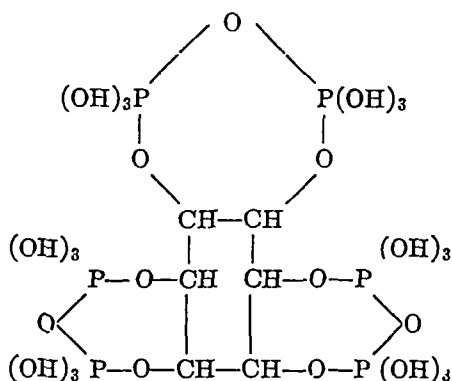
⁸ *Atti R. Accad. dei Lincei Roma* (5), **viii**, 1, p 64

⁹ *Compt. rend. acad. des sci.*, **cxxxvii**, p 439



and which finds expression in the name "anhydro-oxymethylen di-phosphoric acid"

As is well known the free acid, as well as its salts, is easily split under the influence of dilute mineral acids into inosite and ortho-phosphoric acid. This fact and the discovery by Neuberg¹⁰ that both inosite and phytin yield furfural when distilled with phosphorus pentoxide and phosphoric acid, respectively, lead him to believe that the inosite ring exists already formed in phytin. In accordance with this view he proposed the following structural formula for the acid



This is just treble the molecular weight of the anhydro-oxymethylen di-phosphoric acid of Posternak

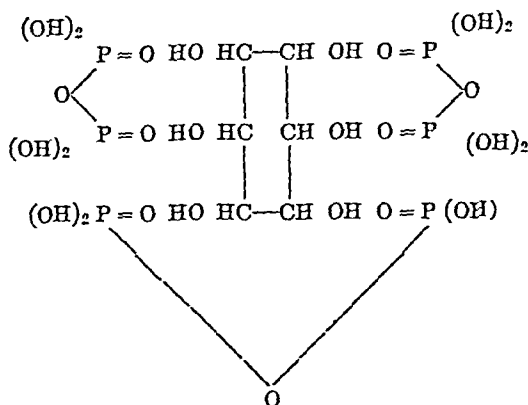
Suzuki and Yoshimura¹¹ considered that phytic acid was the hexa-phosphoric acid ester of inosite

Starkensten¹² believes that phytin represents a complex pyrophosphoric acid compound with inosite and he proposes the following constitutional formula

¹⁰ *Biochem Zeitschr*, ix, pp 551 and 557

¹¹ *Bull Coll of Agric Tokyo*, vii, p 495

¹² *Biochem Zeitschr*, lxx, p 56



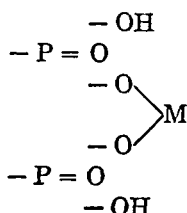
Vorbrodt (*loc cit*) proposes still another formula

It is impossible at the present time to decide definitely between any of the above constitutional formulas, as the substance has not yet been synthesized in the laboratory.

As represented by the empirical formula, $C_6H_{24}O_{27}P_6$, phytic acid corresponds to a hexa-phosphoric acid ester of inosite plus $3H_2O$, $C_6H_6O_6[PO(OH)_2]_6 + 3H_2O$

At present it is impossible to say whether the compound represents a pyrophosphate or if the water is linked in some other way. That the acid contains twelve acid (OH) groups as expressed in the formula of Starkenstein, which would also be the case if it were a hexa-phosphoric acid ester of inosite, and not eighteen (OH) groups as in the formula of Neuberg, seems certain, for in no case have we been able to prepare any salt in which more than twelve H- valences were replaced by bases.

As observed by Starkenstein only one-half of the twelve (OH) groups are particularly reactive. This finds expression in the fact that the barium salt obtained from acid solutions contains only 3Ba to 6P. As suggested by the above author, it is probable that these reactive hydroxyls are adjacent but linked to different phosphoric-acid residues. The salts with binary bases would then be represented by the following



A further confirmation of this is found in the fact that the tri-barium phytate as well as other similar salts of phytic acid with binary bases are strongly acid in reaction

The presence of only eight acid (OH) groups, however, can be shown by titrating an aqueous solution of the acid with decinormal sodium hydroxide using phenolphthalein as indicator Patten and Hart (*loc cit*) who titrated with decinormal barium hydroxide using phenolphthalein as indicator obtained results agreeing with a hexa-barium salt

Of special interest in connection with the constitution of phytin are the phosphoric acid esters of inosite

Neuberg and Kretschner¹³ report obtaining a poly-phosphoric acid ester of inosite by their method of preparing phosphoric acid esters of the carbohydrates and glycerine, that is, by the action of phosphorus oxychloride The product however, could not be obtained pure as it was found impossible to separate it from the inorganic phosphates

Contardi¹⁴ claims to have prepared the hexa-phosphoric acid ester of inosite by heating inosite with an excess of phosphoric acid in a stream of carbon dioxide to 160° to 165°C The product was purified as the barium salt and after decomposing the latter with sulphuric acid the free ester was obtained, which he describes as identical with phytic acid The same author¹⁵ claims to have prepared poly-phosphoric acid esters of mannite, quercite and glucose by the same method

Carré,¹⁶ however, repeating these experiments found that the products described by Contardi were merely mixtures of free phosphoric acid and the polyhydric alcohols in question together with

¹³ *Biochem Zeitschr*, **XXXVI**, p 5

¹⁴ *Atti R Accad dei Lincei Roma*, (5), **XX**, 1, p 23

¹⁵ *Ibid*, p 823

¹⁶ *Bull soc chim de France*, (4), **1**, p 195

their decomposition products mixed with some monobarium phosphate

Many fruitless efforts have been made in this laboratory to synthesize phytic acid and the hexa-phosphoric acid ester of inosite. All experiments in this direction lead only to the tetra-phosphoric acid ester of inosite, $C_6H_6(OH)_2O_4 [PO(OH)_2]$.

The method of Contardi was modified to the extent that inosite, either dry or with water of crystallization, was heated with phosphoric acid, previously dried at $100^\circ C$ to constant weight, in vacuum to a temperature of 140° to $160^\circ C$ for about two hours.

The same product, viz., the tetraphosphoric ester was obtained whether the phosphoric acid was present in large or small excess above six molecules of H_3PO_4 to one molecule of inosite. When it was present in less quantity than this, however, for instance one molecule of inosite to three molecules of H_3PO_4 , then a mixture of esters was formed. It was found impossible to separate these products completely owing to the fact that they possess about the same solubility.

The tetraphosphoric ester is most conveniently isolated by means of its barium salt. The separation of the ester from the excess of the phosphoric acid or barium phosphate succeeded because its barium salt is much less soluble in dilute alcohol acidified with hydrochloric acid than is barium phosphate.

The new ester is a well characterized compound, very similar in appearance and reactions to phytic acid. By heating with acids, inosite and phosphoric acid are regenerated. It gives a white precipitate with the ordinary molybdate solution, and with excess of silver nitrate a white precipitate is also produced. These reactions are identical with those of phytic acid.

The inosite used in these experiments was prepared from the crude magnesium compound previously mentioned and carefully purified by recrystallization.

The reason why phytic acid could not be obtained by the action of phosphoric acid on inosite is no doubt to be found in that it is not a simple ester but a complex compound as suggested by Starkenstein. It is, however, difficult to understand why the hexa-phosphoric ester was not obtained by this method. The only explanation that can be offered is that under the conditions of these experiments it is not stable.

One reason alleged by Starkenstein for considering phytin a pyrophosphate is based upon its giving a white precipitate with silver nitrate. This is certainly a characteristic reaction of pyrophosphates. Yet the tetraphosphoric ester gives a pure white precipitate with the same reagent. As the ester cannot be in the form of a pyrophosphate the fact that phytic acid gives the same colored silver compound is not necessarily an indication that it represents a pyrophosphate compound.

The phytic acid used in these experiments was prepared from products obtained from two different sources. The starting material in one case was a calcium phytate imported from Europe, the other was a crude natural magnesium organic phosphorus compound extracted in this country and kindly supplied us by Dr Carl S. Miner of Chicago.

As shown by the analyses of the carefully purified salts and of the free acid, these two preparations were identical and they were also identical with the product described as phytic acid by Posternak and other investigators.

EXPERIMENTAL PART

Tri-barium phytate

The commercial phytin was purified for analysis by means of the barium salt. Thirty grams calcium phytate were dissolved in a small quantity of 0.5 per cent hydrochloric acid, diluted to about 2 liters with water and a concentrated solution of 30 grams barium chloride was added. The precipitate was dissolved without filtering by the addition of just sufficient dilute hydrochloric acid. It was then precipitated by adding barium hydroxide to faintly alkaline reaction. The mixture was then acidified with acetic acid and after standing over night was filtered and well washed in water. It was re-precipitated in the same manner three times. After finally filtering and washing in water the substance was dissolved in about 1 liter of 0.5 per cent hydrochloric acid, filtered and the filtrate precipitated by adding a like volume of alcohol. After repeating this operation the substance was filtered, washed free of chlorides with 50 per cent alcohol and finally washed in alcohol and ether and dried in vacuum over sulphuric acid.

The product so obtained was a light, perfectly white semi-crystalline or amorphous powder. Placed on moist litmus paper, it showed a strong acid reaction. It is very slightly soluble in water, slightly soluble in acetic acid and readily soluble in mineral acids. For analysis the substance was dried at 130°C

0.2728 gram substance gave 0.0352 gram H_2O and 0.0643 gram CO_2
 0.2763 gram substance gave 0.1749 gram $BaSO_4$ and 0.1675 gram $Mg_2P_2O_7$
 0.1909 gram substance gave 0.1206 gram $BaSO_4$ and 0.1154 gram $Mg_2P_2O_7$
 For $C_6H_7O_9[(PO_3H)_2Ba]_2 = 1120$
 Calculated C = 6.42, H = 1.60, P = 16.60, Ba = 36.78 per cent
 Found C = 6.42, H = 1.44, P = 16.89, Ba = 37.25 per cent
 P = 16.85, Ba = 37.17 per cent

The barium salt prepared in the same manner from a natural crude magnesium organic phosphorus compound gave the following result on analysis

0.2057 gram substance gave 0.0273 gram H_2O and 0.0480 gram CO_2
 0.1422 gram substance gave 0.0886 gram $BaSO_4$ and 0.0841 gram $Mg_2P_2O_7$
 Found C = 6.36, H = 1.48, P = 16.48, Ba = 36.66 per cent

The two salts are therefore identical

Crystallized tri-barium phytate

One gram purified phytic acid was dissolved in 10 cc water and 4 grams of the above mentioned tri-barium phytate added. It was filtered from traces of undissolved particles and allowed to stand for two days at room temperature. The substance has then separated as a heavy crystalline powder of irregular form. From less concentrated solutions the substance separates in small, needle-shaped crystals.

The substance was filtered, washed well in water and finally in alcohol and ether and dried in the air. For analysis it was dried at 120°C

0.1972 gram substance lost 0.0153 gram H_2O
 0.2028 gram substance gave 0.1251 gram $BaSO_4$ and 0.1216 gram $Mg_2P_2O_7$
 Found P = 16.71, Ba = 36.30 per cent
 Calculated for 5 H_2O 7.44, Found 7.75 per cent

Penta-barium phytate

This salt is obtained on neutralizing a solution of the tri-barium phytate in 0.5 per cent hydrochloric acid with barium hydroxide and then acidifying with acetic acid. The precipitate was filtered, washed thoroughly in water, alcohol and ether and dried in vacuum over sulphuric acid.

The product was a white amorphous powder. For analysis the substance was dried at 130°C.

0.2970 gram substance gave 0.0307 gram H_2O and 0.0500 gram CO_2
 0.2507 gram substance gave 0.2080 gram $BaSO_4$ and 0.1207 gram $Mg_2P_2O_7$
 0.1856 gram substance gave 0.1543 gram $BaSO_4$ and 0.0899 gram $Mg_2P_2O_7$
 For $C_6H_{14}O_{27}P_6Ba_5 = 1391$
 Calculated C = 5.17, H = 1.00, P = 13.37, Ba = 49.37 per cent
 Found C = 4.59, H = 1.15, P = 13.42, Ba = 48.82 per cent
 P = 13.50, Ba = 48.92 per cent

Penta-barium ammonium phytate

When the tri-barium phytate is digested in dilute ammonia it is transformed into the penta-barium ammonium salt and ammonium phytate. The latter product, however, was found to contain some barium.

Two grams of the analyzed tri-barium phytate were digested for two hours in 25 cc. of 2.5 per cent ammonia, filtered and washed in dilute ammonia and finally in alcohol and dried in vacuum over sulphuric acid. The product was a heavy white amorphous powder. On moist litmus paper it showed a neutral reaction.

For analysis the substance was dried at 130°C.

0.1509 gram substance gave 0.1205 gram $BaSO_4$ and 0.0762 gram $Mg_2P_2O_7$
 0.1747 gram substance gave 0.0026 gram N (Kjeldahl)¹⁷
 For $C_6H_{14}O_{27}P_6Ba_5(NH_4)_2 = 1425$
 Calculated P = 13.05, Ba = 48.19, N = 1.96 per cent
 Found P = 14.07, Ba = 46.99, N = 1.48 per cent

By evaporating the filtrate from the above to dryness on the water-bath an amber-colored mass remained which after drying at 130°C gave the following result on analysis:

Found P = 20.51, Ba = 6.65, N = 10.48 per cent

¹⁷ This and subsequent nitrogen determinations were made by Mr. M. P. Sweeney.

Penta-magnesium ammonium phytate

Two grams phytic acid were dissolved in 400 cc water and then precipitated by adding excess of magnesia mixture slowly and under constant shaking. After the precipitate had settled the supernatant liquid was decanted, the residue filtered and washed with water until free from chlorides and finally washed in alcohol and ether and dried in vacuum over sulphuric acid.

The product was a fine white amorphous powder and weighed 2.7 grams. It reacts neutral on moist litmus paper. For analysis it was dried at 130°C.

0.1089 gram substance gave 0.0832 gram Mg P₂O₅ for P
 0.1089 gram substance gave 0.0705 gram Mg P₂O₅ for Mg
 0.1248 gram substance gave 0.0039 gram N
 0.0893 gram substance gave 0.0028 gram N } (Kjeldahl)

For C₆H₁₀O₇·P₅Mg₅(NH₄)₂ = 859.5

Calculated P = 21.64, Mg = 14.13, N = 3.25 per cent

Found P = 21.29, Mg = 14.13, N = 3.12 - 3.13 per cent

If the phytic acid is first neutralized with ammonia and then precipitated with magnesia mixture the same product is obtained.

Two grams phytic acid in 400 cc water were neutralized with ammonia, precipitated with excess of magnesia mixture, filtered, washed free of chlorides with dilute ammonia and then in alcohol and dried in vacuum over sulphuric acid. For analysis the substance was dried at 130°C.

Found P = 21.49, Mg = 13.96, N = 3.47, 3.48 per cent

Tetra-cupric di-calcium phytate

To a solution of 2 grams phytic acid in 200 cc water excess of calcium chloride was added and the solution then neutralized with ammonia. The precipitate was just dissolved in dilute hydrochloric acid and the solution precipitated with copper acetate. The bluish-green colored copper compound was filtered off, washed with water until free from chlorides and then in alcohol and dried in vacuum over sulphuric acid.

The dry substance was a light-blue amorphous powder. It is very slightly soluble in water or in very dilute acids, readily soluble

in the ordinary dilute mineral acids. It is readily soluble in 2.5 per cent ammonia with a deep-blue color. In this solution concentrated ammonia or alcohol produces a light-blue colored precipitate.

The compound represents a nearly pure tetra-cupric di-calcium phytate. It contained 0.17 per cent N.

For $C_6H_{12}O_9 (PO_3Cu)_4 (PO_3Ca)_2 = 1036$

Calculated Cu = 24.51, Ca = 7.72, P = 17.95 per cent

Found Cu = 25.58, Ca = 7.69, P = 16.85 per cent

If a slightly acid solution of magnesium ammonium phytate is precipitated with copper acetate a light blue colored copper compound is obtained. After washing and drying it gave the following result on analysis:

Mg = 1.11, Cu = 34.27, N = 0.64 and 0.52, P = 15.66 per cent

This compound is exceedingly soluble in dilute and concentrated ammonia. By the careful addition of alcohol to the ammoniacal solution a substance separates in light blue colored crystals on standing. This is evidently a complex copper-ammonium salt but it was not further examined.

Phytic acid

This was prepared after the method of Patten and Hart (*loc cit*). The analyzed tri-barium salt was decomposed with the calculated quantity of decinormal sulphuric acid. After removing the barium sulphate, the solution was precipitated with copper acetate. The copper compound was decomposed with hydrogen sulphide, the copper sulphide filtered off, the filtrate concentrated in vacuum and finally dried in vacuum over sulphuric acid. The products obtained from both the calcium phytate and the magnesium compound were light amber colored, very thick liquids and corresponded in all respects with the body described by other investigators as phytic acid.

For analysis the substance was dried at 130°C.

a From calcium phytate

0.3193 gram substance gave 0.0917 gram H_2O and 0.1238 gram CO_2

0.1505 gram substance gave 0.1424 gram $Mg \cdot P \cdot O_7$

b From the magnesium compound

0.2789 gram substance gave 0.0804 gram H₂O and 0.1101 gram CO

0.1236 gram substance gave 0.1160 gram Mg₂P₂O₇

For C₆H₄O₄ · P₂O₅ = 714

	Calculated	Found I	Found II
C	10.08	10.57	10.76
H	3.36	3.21	3.22
P	26.05	26.37	26.16

Titrated against decinormal sodium hydroxide using phenolphthalein as indicator the following results were obtained

0.2648 gram acid required 30.7 cc $\frac{N}{10}$ NaOH

Calculated for 8NaOH 29.65 cc

0.1593 gram acid required 18.60 cc $\frac{N}{10}$ NaOH

Calculated for 8NaOH 17.60 cc

Inositol from the crude magnesium compound

Twenty-five grams of the air-dried substance, containing 20 per cent of moisture, was heated with 100 cc of 30 per cent sulphuric acid in a sealed tube for about three hours at a temperature of 140°C. Two tubes equally charged were heated at the same time. After cooling the reaction mixture was of dark brown color and a considerable quantity of magnesium salts had crystallized out.

The contents were washed into a beaker, filtered and diluted with water to about 1500 cc. The sulphuric and phosphoric acids and the magnesium were then precipitated by barium hydroxide, filtered and well washed in hot water. The filtrate was evaporated to about 350 cc and the excess of barium removed by carbon dioxide, filtered, the filtrate decolorized with animal charcoal and then evaporated on the water bath to a syrupy consistency. This was taken up in a small quantity of hot water, filtered and alcohol added to the filtrate until a cloudiness was produced. By scratching with a glass rod crystallization began, more alcohol was then added and the mixture placed in the ice-chest over night. After filtering and washing in alcohol and ether and drying in the air the product weighed from 5.1 to 5.4 grams. From the mother liquor a further quantity of crystals from 0.4 to 0.6 gram could be obtained on the addition of ether and allowing to stand for twenty-four hours in the cold.

For purification the raw product was dissolved in six parts of water and again brought to crystallization by the addition of alcohol as before. It was then obtained in large, thin, colorless plates.

It gave the reaction of Scherer. The dried substance melted at 220°C (uncorrected).

Dried at 100°C , 0.4136 gram substance lost 0.0669 gram H_2O and 0.1600 gram lost 0.0258 gram H_2O .

The dried substance was analyzed

0.1342 gram substance gave 0.0791 gram H_2O and 0.1981 gram CO_2

For $\text{C}_6\text{H}_6(\text{OH})_6 = 180$

Calculated C = 40.00, H = 6.66, $2\text{H}_2\text{O} = 16.66$ per cent

Found C = 40.26, H = 6.59, $2\text{H}_2\text{O} = 16.17 - 16.12$ per cent

This substance was used in subsequent experiments with phosphoric acid. Some 40 grams of inosite were prepared in this way.

Tetra-phosphoric acid ester of inosite

Crystallized inosite (4.32 grams, 2 molecules) was powdered and mixed in a distillation flask with 24 grams phosphoric acid (about 24 molecules or double the quantity required to form the hexaphosphoric ester). The acid had been previously dried at 100°C to constant weight. The flask was connected with the vacuum pump and heated in an oil bath to 140° to 160°C for about two hours. By 120° water began to come over and the reaction was practically complete at the end of one hour. After cooling the reaction mixture was a thick, reddish-brown colored, nearly solid mass. This was dissolved in about 1 liter of water and a solution of 40 grams of barium chloride in 400 cc of water was added. The barium salt of the ester was then precipitated by the addition of about 1 liter of alcohol.

A solution containing phosphoric acid and barium chloride in the same dilution as above remains perfectly soluble on the addition of a like volume of alcohol.

The voluminous flaky precipitate was filtered off at once and thoroughly washed in $33\frac{1}{3}$ per cent alcohol.

For purification the substance was dissolved in 700 cc of 0.5 per cent hydrochloric acid, filtered from slight insoluble residue, the filtrate diluted with 500 cc of water, some barium chloride added and then precipitated by the addition of a like volume of alcohol. This was repeated a second time. The substance was

then dissolved in 500 cc of 0.5 per cent hydrochloric acid, precipitated by adding barium hydroxide to slightly alkaline reaction, then acidifying with hydrochloric acid and adding 500 cc alcohol. After filtering and washing as before the substance was again twice precipitated from 0.5 per cent hydrochloric acid solution with alcohol and finally washed in 50 per cent alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product weighed 8.9 grams. It was a white voluminous amorphous powder. On moist litmus paper it showed a strong acid reaction. The solubility of the product was practically the same as for the tri-barium phytate.

For analysis it was dried at 100° and 130°C

0.3252 gram substance lost 0.0231 gram H₂O
 0.2697 gram substance gave 0.0442 gram H₂O and 0.0878 gram CO
 0.2038 gram substance gave 0.0300 gram H₂O and 0.0685 gram CO₂
 0.2482 gram substance gave 0.1505 gram BaSO₄ and 0.1434 gram Mg₃P₂O₇
 0.1833 gram substance gave 0.1108 gram BaSO₄ and 0.1075 gram Mg₃P₂O₇
 0.1776 gram substance gave 0.1074 gram BaSO₄ and 0.1038 gram Mg₃P₂O₇
 For C₆H₈(OH)₂O₄[(PO₃H)₃Ba]₃ = 770.7
 Calculated C = 9.34, H = 1.55, P = 16.08, Ba = 35.64 per cent
 Found C = 8.87, H = 1.83, P = 16.10, Ba = 35.68 per cent
 C = 9.16, H = 1.64, P = 16.34, Ba = 35.57 per cent
 P = 16.29, Ba = 35.58 per cent
 Calculated for 4 H₂O 8.55, Found 8.64 per cent

Another lot prepared by heating 1.80 grams dry inositol (1 molecule) with 7.9 grams dry phosphoric acid (about 8 molecules) and isolated in the same manner gave the following results on analysis

0.2879 gram substance lost 0.0240 gram H₂O
 The dried substance was analyzed
 0.2639 gram substance gave 0.0452 gram H₂O and 0.0936 gram CO₂
 0.1480 gram substance gave 0.0866 gram BaSO₄ and 0.0846 gram Mg₃P₂O₇
 0.1632 gram substance gave 0.0959 gram BaSO₄ and 0.0933 gram Mg₃P₂O₇
 Found C = 9.67, H = 1.91, P = 15.93, Ba = 34.43 per cent
 H₂O = 8.33, P = 15.93, Ba = 34.58 per cent

A third lot prepared by heating 1.80 grams dry inositol (1 molecule) with 5.88 grams dry phosphoric acid (6 molecules) and isolated in the same manner as before gave the following

C = 9.69, H = 1.75, P = 16.06, Ba = 36.33 per cent

It is apparent therefore that in each of the above experiments the same compound was produced

The free tetra-phosphoric ester

About 5 grams of the purified barium salt was decomposed by digesting it with the calculated quantity of decinormal sulphuric acid. After removing the barium sulphate the solution was precipitated with excess of copper acetate. The copper precipitate was filtered, thoroughly washed with water, suspended in water and decomposed with hydrogen sulphide. The copper sulphide was removed by filtration, the filtrate concentrated in vacuum and finally dried in vacuum over sulphuric acid until it was of a thick, syrupy consistency.

For analysis the substance was dried at 130°C

0.3020 gram substance gave 0.0933 gram H_2O and 0.1577 gram CO_2

0.1605 gram substance gave 0.01387 gram $Mg_2P_2O_7$

For $C_6H_6(OH)_2O_4[PO(OH)_2]_4 = 500$

Calculated C = 14.40, H = 3.20, P = 24.80 per cent

Found C = 14.24, H = 3.45, P = 24.09 per cent

0.1663 gram substance required 16.5 cc decinormal sodium hydroxide using phenolphthalein as indicator. This corresponds to five acid (OH) groups.

Calculated for $5NaOH$ 16.63 cc

Properties of the free ester

The concentrated aqueous solution of the ester is very similar to phytic acid. It is a very thick amber-colored liquid of sharp acid, slightly astringent taste and strong acid reaction. On longer keeping in the desiccator over sulphuric acid it becomes hard and brittle and may be powdered. It is then very hygroscopic.

The dry substance is slowly but completely soluble in alcohol, readily soluble in water.

The concentrated aqueous solution gives a white precipitate with silver nitrate in excess which dissolves on largely diluting with water. The precipitate is readily soluble in ammonia, dilute nitric, sulphuric and acetic acids, insoluble in glacial acetic acid.

With ferric chloride it gives a white or faintly yellowish precipitate which is very sparingly soluble in acids.

With lead acetate a white precipitate is produced, readily soluble in dilute nitric acid but sparingly soluble in acetic acid.

With barium chloride it gives a white precipitate slightly soluble in acetic acid but readily soluble in hydrochloric and nitric acids.

Calcium chloride does not give a precipitate but on heating the calcium salt is thrown down as a white precipitate which redissolves on cooling

Magnesium salts do not cause a precipitate and on heating the solution merely turns cloudy, on cooling it clears up again

With the ordinary molybdate solution it gives in the cold a white voluminous flaky precipitate which slowly turns yellowish in color Phytic acid under the same conditions gives a white precipitate which remains unchanged in the cold On drying at 110° or 130° the substance turns very dark in color

The ester, like phytic acid, fails to give directly the Scherer reaction for inosite

Inosite from the tetra-phosphoric ester

Ten grams of the purified barium salt was heated with 25 cc 30 per cent sulphuric acid in a sealed tube to about 150°C for three hours After precipitating the sulphuric and phosphoric acids with barium hydroxide the inosite was isolated by the usual method and recrystallized from hot dilute alcohol It was filtered and washed in alcohol and ether and dried in the air Yield, 1.52 grams It was obtained in the form of small colorless six-sided plates, free from water of crystallization

The air-dried, water-free substance melted at 221°C (uncorrected)

0.2094 gram substance gave 0.1259 gram H_2O and 0.3033 gram CO_2

0.1360 gram substance gave 0.0827 gram H_2O and 0.1991 gram CO_2

For $\text{C}_6\text{H}_8\text{O}_6 = 180$

Calculated C = 40.00, H = 6.66 per cent

Found C = 39.50, H = 6.72 per cent

C = 39.93, H = 6.80 per cent

As already mentioned, if a mixture of inosite and phosphoric acid is heated when less than six molecules H_3PO_4 are present to one molecule inosite, a mixture of esters is obtained It was found impossible to separate these bodies as barium salts and obtain pure compounds since their solubilities are apparently nearly alike

Dry inosite (3.60 grams, 2 molecules) and 5.88 grams dry phosphoric acid (6 molecules) was heated in a distillation flask as before to 180° to 190° for about two hours, until water ceased coming over The reaction mixture was in the form of a very bulky thin

flaky mass, very brittle and of yellowish-brown color, mixed with some very dark-colored substance. It was broken up with a glass rod and removed from the flask and treated with water in which the dark-colored portion was readily soluble, but the lighter-colored substance was insoluble in this medium. It was powdered in a mortar and thoroughly washed in water and alcohol and dried in vacuum over sulphuric acid.

The substance was apparently insoluble in boiling water, in boiling dilute acids and in glacial acetic acid, also insoluble in alcohol, ether and other organic solvents. After drying at 130° the substance was analyzed.

0.2500 gram substance gave 0.0838 gram H_2O and 0.2085 gram CO_2

0.1500 gram substance gave 0.1145 gram $Mg_2P_2O_7$

0.1542 gram substance gave 0.1178 gram $Mg_2P_2O_7$

Found C = 22.74, H = 3.75, P = 21.28, 21.29 per cent

This agrees approximately with a mono-pyro-phosphoric ester of inosite but the phosphorus is too high.

It was decided to purify it by means of the barium salt. The substance was dissolved by boiling in dilute sodium hydroxide in which it gave a dark amber colored solution. After filtering, it was precipitated with barium chloride, the barium precipitate filtered and washed free of alkali. It was then dissolved in 500 cc 0.5 per cent hydrochloric acid and precipitated by barium hydroxide. After filtering and washing it was repeatedly precipitated with alcohol from 0.5 per cent hydrochloric acid solution until finally a small amount of a white amorphous powder was obtained. After drying at 130° this was analyzed.

0.2028 gram substance gave 0.0412 gram H_2O and 0.0979 gram CO_2

0.2207 gram substance gave 0.0413 gram H_2O and 0.1042 gram CO_2

0.1982 gram substance gave 0.0996 gram $BaSO_4$ and 0.1103 gram $Mg_2P_2O_7$

Found C = 13.16, H = 2.27, P = 15.51, Ba = 29.57 per cent

C = 12.88, H = 2.09

In this compound the relation between the carbon and phosphorus is nearly 6C to 3P which would indicate a tri-phosphoric ester. The substance was, however, far from pure and lack of material prevented any further investigation of this body, which is apparently a mixture of various esters.

ON THE PRESENCE OF ACTIVE PRINCIPLES IN THE THYROID AND SUPRARENAL GLANDS BEFORE AND AFTER BIRTH

By FREDERIC FENGER

(From the Chemical Research Laboratory in Organotherapeutics of Armour
and Company, Chicago)

(Received for publication, April 4, 1912)

From a chemical standpoint the two best known of the ductless glands are the thyroid and the suprarenals. The active principle of the suprarenals has been separated in pure crystalline form and we have well defined methods for its identification and quantitative estimation.

The activity of the thyroid gland is measured by its iodine content. Sajous, in his recent work,¹ calls attention to the fact that it is absolutely established that an iodine compound is the active agent of the thyroparathyroid secretions. Reid Hunt states² that the active principle of this gland is associated with iodine and that the therapeutic activity of the various preparations from this gland is proportional to the amount of iodine in thyroid combination present therein, and that consequently the iodine may be used as a basis for standardization of such preparation. Beebe³ confirms this statement.

It has been stated that the thyroid gland of new-born animals⁴ does not contain any iodine.

To the writer it appeared unlikely that either the thyroid or suprarenal glands should be free from their active principles up to the time of birth. We know that the ductless glands inject their secretions into the circulatory and lymphatic system.⁵ If, there-

¹ *The Internal Secretions and the Principle of Medicine*, 1, p. 156, 1911

Journ Amer Med Assoc, Oct 24 1908, p. 1386

² *Ibid*, lv, p. 658, March, 1911

³ *Ibid*, lv, p. 1983, Dec 3, 1910

⁵ *Ott Internal Secretions*, 1910, p. 93

fore, the secretions of these glands are necessary, not merely for the maintenance of life and healthy metabolism, but also to govern the growth of the young animal, we might reasonably expect to find these glands active not merely at time of birth but also in the fetus, especially as these glands only produce internal secretions which, as far as we know, do not enter the alimentary tract. These considerations led the writer to conduct the experiments described below.

The lack of available material, *i e*, normal healthy glands, would prevent a thorough and practical investigation of this subject as far as the human body is concerned. Of the domestic animals, cattle are best adapted for such experiments.

For this series of experiments, which were carried out during March, 1912, thyroids as well as suprarenals were used and in the case of cattle, four stages of age were selected, namely, the fetus about three months old, the fetus about eight months old, young suckling calves six to eight weeks old, and full-grown cattle.

In general the suprarenal glands from beef, hog and sheep seem to be of fairly uniform and proportional size and color. The thyroid glands on the other hand, varied enormously both in size and color. This is especially true of beef and sheep. An investigation is now being carried on in order to look into this matter more thoroughly, and the results will be reported in a later paper. In this paper only normal-sized healthy glands are considered. It should be borne in mind that the period of gestation for cows is nine to nine and one-half months, for sheep five months, and for hogs four months.

The method of preparation was briefly as follows.

The fresh glands were trimmed and weighed, minced, dried at 35° to 50°C to constant weight, and freed from fat by extraction with petroleum ether.

All determinations were made in duplicate on composite samples of the number of glands specified in the tabulation. The thyroid and suprarenal glands were obtained from the same animals in case of all sheep and hog fetus and three months old beef fetus. The thyroid glands from eight months old beef fetus as well as those from suckling calves and all the grown animals were not out of the same animals as the suprarenal glands of corresponding age.

The iodine determinations were made according to Hunter's excellent method ⁶

The active principle of the suprarenal glands was determined colorimetrically according to the iodic acid method suggested by Hale and Seidell⁷ with the exception that samples of desiccated beef, hog and sheep suprarenals of known physiological strength were used for comparison instead of the proposed permanent standards

The results are given in the table on page 492 Those obtained on the sheep and hog glands are somewhat incomplete partly due to the fact that the glands in the fetus are very small, and also because suckling lambs and pigs are not commonly used for human food, and consequently obtainable only with great difficulty

The results obtained above indicate definitely that the thyroid gland of these animals contains iodine, not merely at time of birth but long before

Since the amount of iodine in the thyroid is an indication of the relative activity of this gland there is evidently a gradual rise in activity of the gland in the fetus, and this activity is increased rapidly shortly after birth, reaching its maximum in the young growing animal

The iodine content of the glands from the full-grown animals is very low This is, however, not unusual as the iodine content varies considerably The glands were collected during the same period as the glands from the various fetus and the analyses are given here for comparative purposes only

The active principle of the suprarenals is also present in the fetus long before maturity, and in comparatively higher quantities than in the full-grown animal

As time permits and opportunities present themselves, it is the writer's intention to confirm these results, and to carry on further and more extended investigations along these lines, in the hope that the data so obtained may throw further light on the activity of the ductless glands

In conclusion it may be stated that in all his experience with the thyroid glands from beef, hog and sheep, the writer has never found a sample of known origin that did not contain iodine

⁶ This Journal, vii, p 321, 1910

⁷ Amer Journ of Pharm, Dec, 1911, p 551

Thyroid and Suprarenal Glands

	BEEF THYROIDS				SHEEP THYROIDS		HOG THYROIDS	
	Fetus Three Months Old	Fetus Seven to Eight Months Old	Suckling Calves Six to Eight Weeks Old	Full-Grown Cattle	Fetus Three to Four Months Old	Full-Grown Sheep	Fetus Seventy Days Old	Full-Grown Hogs
Number of glands	6	24	16	200	40	200	18	200
Average weight per gland (both lobes), grams	1 6	9 0	10 0	59 0	1 3	8 9	0 3	8 8
Moisture, <i>per cent</i>	84 1	82 3	74 3	72 6	82 3	78 3	85 9	67 2
Soluble in petroleum ether, <i>per cent</i>	1 1	1 2	7 0	8 2	1 7	4 1	trace	13 0
Desiccated fat-free gland, <i>per cent</i>	14 8	16 5	18 7	19 2	16 0	17 6		19 8
Iodine in fat-free gland, <i>per cent</i>	0 08	0 19	0 32	0 03	0 10	0 06	0 09	0 27
	BEEF SUPRARENALS				SHEEP SUPRARENALS		HOG SUPRARENALS	
Number of glands	12	20	30	200	38	200	25	200
Average weight per gland, <i>grams</i>	0 3	0 9	2 2	12 0	0 16	1 3	0 05	3 2
Moisture, <i>per cent</i>	86 0	83 0	78 3	74 2	84 5	73 7	84 9	72 7
Soluble in petroleum ether, <i>per cent</i>		2 2	2 8	6 3	1 2	5 0		8 5
Desiccated fat-free gland, <i>per cent</i>		14 8	18 9	19 5	14 3	21 3		18 8
Epinephrine in desic- cated fat-free gland, <i>per cent</i>	3 5	3 5	4 0	2 5	2 0	1 0	1 75	1 20

A NEW METHOD FOR THE DETERMINATION OF TOTAL NITROGEN IN URINE

BY OTTO FOLIN AND CHESTER J. FARMER

(From the Biochemical Laboratory of Harvard Medical School, Boston)

(Received for publication, April 12, 1912)

No one analytical method has done so much to further metabolism investigations as Kjeldahl's method for the determination of total nitrogen. While applicable to all kinds of nitrogenous products of interest to the biochemist it has proved particularly serviceable in urine analysis. In its modern modifications it is one of the most rapid, convenient and accurate methods we have. At first sight it might therefore seem a thankless and superfluous task to attempt to find a substitute for such an admirable tool for research. Our original idea in attempting to find another method for the determination of total nitrogen in urine was, however, to fill a gap which Kjeldahl's method does not fill, it just falls short of being suitable for clinical work except in the very best hospital laboratories.

Our original purpose was to decompose an accurately measured minute quantity of urine by means of sulphuric acid and mercury. Then, making use of the mercury for the formation of Nessler's reagent, produce the color reaction directly in the digestion mixture. We should thus have had an ideal clinical method. Because of the difficulties encountered in trying to overcome the turbidity produced on Nesslerizing the ammonia in such mixtures we have temporarily at least abandoned that scheme.

In principle our new method may be described as a microchemical method based on the Kjeldahl-Gunning process for decomposing nitrogenous materials and on the methods of Nessler and of Folin for the determination of ammonia. Rapidity in every stage of the process is secured by reducing the amount of urine taken for an analysis. In the ordinary Kjeldahl determination from 30 to 100 mgms of nitrogen is used while we work with only about 1 mgm. To many it may at first seem questionable whether a considerable element of error is not inevitably intro-

duced by reducing the amount of urine taken for a determination to the quantitative nitrogen level that is employed in water analysis. The accuracy of analytical results depends, however, far more on the nature of the chemical reactions employed than on the quantity of material actually weighed or measured. By means of suitable so-called Ostwald pipettes¹ 1 cc. can easily be measured to within an accuracy of about 0.1 per cent and so far as this one phase of the work is concerned nothing is gained by using 5 or 10 cc.² The only precaution called for in the use of these pipettes is to let them drain against the sides of the test tube for ten seconds and then blow them out clean so that nothing is left behind in the tip. One cubic centimeter of urine contains ordinarily from 5 to 20 mgms. of nitrogen. For colorimetric work with Nessler's reagent even 1 cc. of urine is therefore much more than can be advantageously used, although we have improved the Nesslerization process so that several milligrams of ammonia can be satisfactorily determined colorimetrically. We occasionally used 1 cc. of undiluted urine and titrate the ammonia, as in the Kjeldahl method (see p. 500). For the colorimetric determination, however, we invariably dilute the urine until 1 cc. contains from 0.75 to 1.5 mgms. of nitrogen.

The method, as we have now used it in this laboratory for nearly two years, is as follows:

Five cubic centimeters of urine is measured into a 50 cc. measuring flask if the specific gravity of the urine is over 1.018, or into a 25 cc. flask if the specific gravity is less than 1.018. The flask is filled to the mark with water and inverted a few times to secure thorough mixing. One cubic centimeter of the diluted urine is then measured into a large test tube made of Jena glass (size 20 to 25 mm. by 200 mm.). To the urine in the test tube add 1 cc. of concentrated sulphuric acid, 1 gram of potassium sulphate, 1 drop of 5 per cent copper sulphate solution and a small, clean quartz pebble (to prevent bumping). Boil over a micro-burner³

¹ Ostwald-Luther *Physiko-Chemische Messungen*, 2d ed., p. 135

² From Eimer and Amend can now be obtained the kind of pipettes which we use in our work. The only difference between them and Ostwald's is that they are made of thicker glass tubing and the stems are longer.

³ The microburner, No. 2587 Eimer and Amend, is very satisfactory. The flame must of course not be so high as to unduly heat the test tube above the liquid.

for about six minutes, *i e*, about two minutes after the mixture has become colorless. Allow to cool about three minutes until the digestion mixture is beginning to become viscous (it must not be allowed to solidify). Then add about 6 cc of water, at first a few drops at a time, then more rapidly so as to prevent the mixture from solidifying. To the acid solution is then added an excess of sodium hydrate (3 cc of saturated solution) and the ammonia is aspirated by means of a rapid air current into a measuring flask (volume 100 cc) containing about 20 cc of water and 2 cc of $\frac{1}{10}$ hydrochloric acid. The air current used for driving off the ammonia may well be rather moderate for the first two minutes but thereafter for eight minutes should be as rapid as the apparatus can stand.

Now disconnect, dilute the contents in the flask to about 60 cc, and dilute similarly 1 mgm of nitrogen, in the form of ammonium sulphate (see p 496), to about the same volume in a second measuring flask. Nesslerize both solutions as nearly as possible at the same time with 5 cc of Nessler's reagent diluted immediately beforehand with about 25 cc of water. (Five cubic centimeters of Nessler's reagent gives the maximum color with 1 to 2 mgms of ammonia and when diluted as indicated turbidity is avoided.) The color produced does not reach the maximum till the end of about half an hour but the increase is small and is immaterial to the result when the reagent is added as described, *i e*, practically simultaneously to the standard and to the unknown ammonium salt solution. The two flasks are therefore at once filled to the mark with distilled water, mixed, and the relative intensity of the colors is determined by means of a colorimeter.

In making colorimeter readings it is important to adjust the unknown to that of the standard both from above and from below the level of the latter. If the color is adjusted only from above one is apt to consider the two fields equal when the unknown is still too dark and if from below the reverse is the case. This is true for any kind of comparison of colors or of light intensity.

In all of our work we have used the Duboscq colorimeter. A much cheaper instrument designed by Professor White of Harvard University primarily for use in the iron and steel industry we have also found fairly serviceable.

The calculation of the result is simple. The reading of the standard divided by the reading of the unknown gives the nitrogen in milligrams in the volume of urine taken.

It has taken us a long time to devise the above simple procedure for the determination of nitrogen in urine

1 At first we were unable to secure satisfactory results because our standard ammonium sulphate solutions were not trustworthy, notwithstanding the fact that they gave practically theoretical results when their ammonia was determined by distillation and titration Other salts of ammonia were even worse than the sulphate Because of this fact our results were too high and we were led to suspect the presence of ammonia or other nitrogenous products in our reagents The error was due to pyridine bases present in all ammonium salts These bases titrate like ammonia but do not give the reaction with Nessler's reagent

Pure ammonium sulphate can be made by decomposing a high grade ammonium salt with caustic soda and passing the ammonia gas into pure sulphuric acid by means of the air current The salt so obtained is precipitated by the addition of alcohol, is redissolved in water and again precipitated with alcohol and finally dried in a desiccator over sulphuric acid ⁴

2 Another difficulty which we had to overcome was the frequency with which the Nessler reagent produced turbidity instead of clear solutions In water analysis the amounts of ammonia are very small though even in water analysis failures due to this cause are not uncommon Winkler's modification of Nessler's solution consisting in substituting mercuric iodide for the chloride in the preparation in the reagent represents an effort to prevent turbidity The remedy which we finally discovered consists, as indicated in the above description, in diluting the reagent with about five volumes of water When so diluted the reagent can literally be dumped into the ammonia solution even when as much as 2 mgms is present, and the result is a deep wine color but no turbidity If turbidity does occur it is because the Nessler solution is not sufficiently diluted with water before being added to the ammonium salt solution To secure the maximum color, the reagent is, however, best added about one third at a time The diluted Nessler-Winkler solution does not keep for more than a few minutes, a brick red precipitate settling out, hence the dilution should not take place until just before it is needed When

⁴ Dr R L Emerson, Boston, now prepares our ammonium sulphate for us in the manner described

once added to the ammonium salt solution, even though the amount of ammonia present be very small, the decomposition of the reagent is checked

3 As in nearly all other quantitative colorimetric comparisons it is here necessary for accurate work that the amount of color produced in the unknown should be reasonably near that of the standard (see, however, p 534) Using 1 mgm of nitrogen as a standard, the unknown should contain between 0.75 and 1.5 mgms If much more or considerably less nitrogen is present the colorimetric readings become less accurate The standard can be set at any desired depth, but 20 mm represents the standards we ordinarily use (with the Duboscq colorimeter)

The color is extremely easy to read quantitatively Diffused daylight is by far the best but it is possible to get fairly reliable readings with an electric light by interposing a sheet of smooth white paper between the source of light and the colorimeter, careful adjustment of the instrument so as to secure equal illumination in both fields is, however, imperatively necessary when artificial light is used

4 In order to remove the ammonia from the digestion mixture in the shortest possible time the volume of the solution should be kept at a minimum There is danger of loss of ammonia, however, if this attempt to keep down the volume is carried too far, for when sodium or potassium sulphate settles out, as it will do immediately on adding the alkali if the volume of water previously added is too small, it carries down more or less ammonium sulphate The sulphates must therefore not begin to come down until the air current has already removed the greater part of the ammonia, *i e*, until it has been going a couple of minutes After this time and when the solution is getting cold, more or less sulphate invariably settles out but this does no harm It is of course perfectly feasible to dilute the digestion mixture with more than the 6 cc of water prescribed above and thus entirely avoid the formation of any precipitate but the conditions described are the most advantageous and when followed, every trace of ammonia present in the digestion mixture will be removed by a strong air current in eight to ten minutes

5 The most convenient method for adding 3 cc of saturated sodium hydroxide solution to the warm digestion mixture is to

suck it up into the glass tube which goes to the bottom of the test tube and through which the air is forced through the alkaline mixture ⁵ By means of a short rubber tube and a pinch cock the tube is temporarily used as a pipette for the transference of the alkali

6 In Fohn's air current method the ammonia was made to pass through a filter consisting of a calcium chloride tube filled with cotton wool In this case no such filter is needed and is less desirable because of the small amounts of ammonia involved A cheap (unmarked) 5 cc pipette is used instead as shown in the drawings

It is, however, highly desirable, if not necessary, to prevent the concentrated alkaline sulphate solution from splashing up into the tube (made from the pipette) for if much gets there a little will creep up along the sides of the tube and get into the receiver Since the air current is to be a rapid one this is likely to happen if nothing is done to prevent it A simple yet very effective trap is shown in the drawings below It consists of a circular piece of rubber cut out of a two-holed rubber stopper or rubber matting about a quarter of an inch thick and is slipped on to the glass tube which reaches to the bottom of the test tube It should be small enough to easily get into the test tube yet large enough to prevent the splash from striking the opening of the exit tube One or two notches are cut into the edges so that the liquid which does get above the trap can easily flow back again without obstructing the air currents

7 In most modern laboratories compressed air is available and where that is the case the air (and ammonia) is pushed through the apparatus This is the most convenient method for isolating the ammonia since it is to be collected in a measuring flask the neck of which is not wide enough for a two-hole rubber stopper The necessary air current can, however, be obtained without much trouble from a good suction pump The air should be washed free from any traces of ammonia it may contain by passing it through a bottle of dilute sulphuric acid When suction is

⁵ It is important that the glass tubes passing through the rubber stopper should not be too large for the holes in the stopper If the latter remains perfectly round the test tube is most easily closed perfectly tight without using undue pressure

employed the ammonia is not absorbed directly in the measuring flask for the reason stated above. It is collected in a second large tube in 2 cc of $\frac{N}{10}$ acid and about 5 cc of water. The ammonium salt solution is then rinsed into the measuring flask with 40 to 50 cc of water and is then Nesslerized as described.

The drawings below illustrate how the apparatus is set up for use (a) with compressed air or a force pump, (b) with a vacuum pump. When the short rubber tube carrying the pinch cock is withdrawn the alkali gets into the digestion mixture. Connection with the air current is then made and the aspiration is begun.

8 The acid in the volumetric flask used as a receiver should be small in amount for with a large excess present the color develops rather more slowly. Two cubic centimeters of tenth normal acid is enough for the retention of 2 mgms of ammonia nitrogen.

In order to secure perfect absorption of the ammonia a glass tube sealed at one end but containing three or four little holes drilled into the tube by means of a hot platinum wire is used. Such a tube can be made in a few minutes and is adequate as a substitute for the special absorption tube used by Folin for the absorption of larger amounts of ammonia.⁶

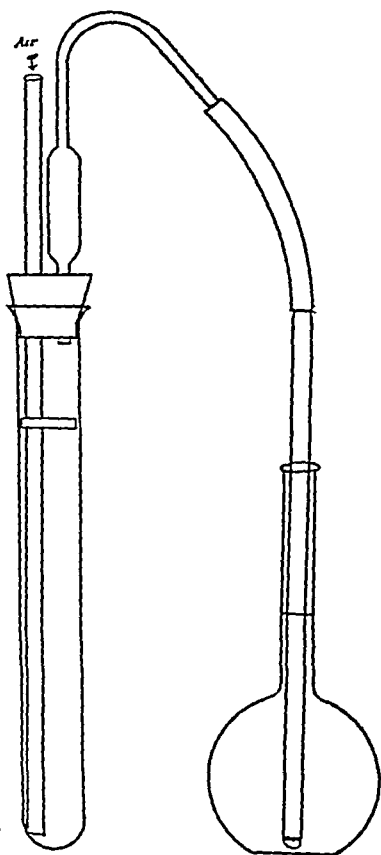


FIG 1 APPARATUS FOR USE WITH COMPRESSED AIR

9 The microchemical method for the determination of nitrogen

⁶ Many seem to have trouble about making holes by means of the hot platinum wire. By having the glass only moderately hot (not hot enough to be soft) and keeping the wire at a white heat all difficulties are avoided.

has been described above exclusively on the basis of colorimetric comparisons with standard ammonium salt solutions. The colorimetric principle is, however, not indispensable. One cubic centimeter of urine previously diluted with an equal volume of water can be decomposed as described above and the ammonia

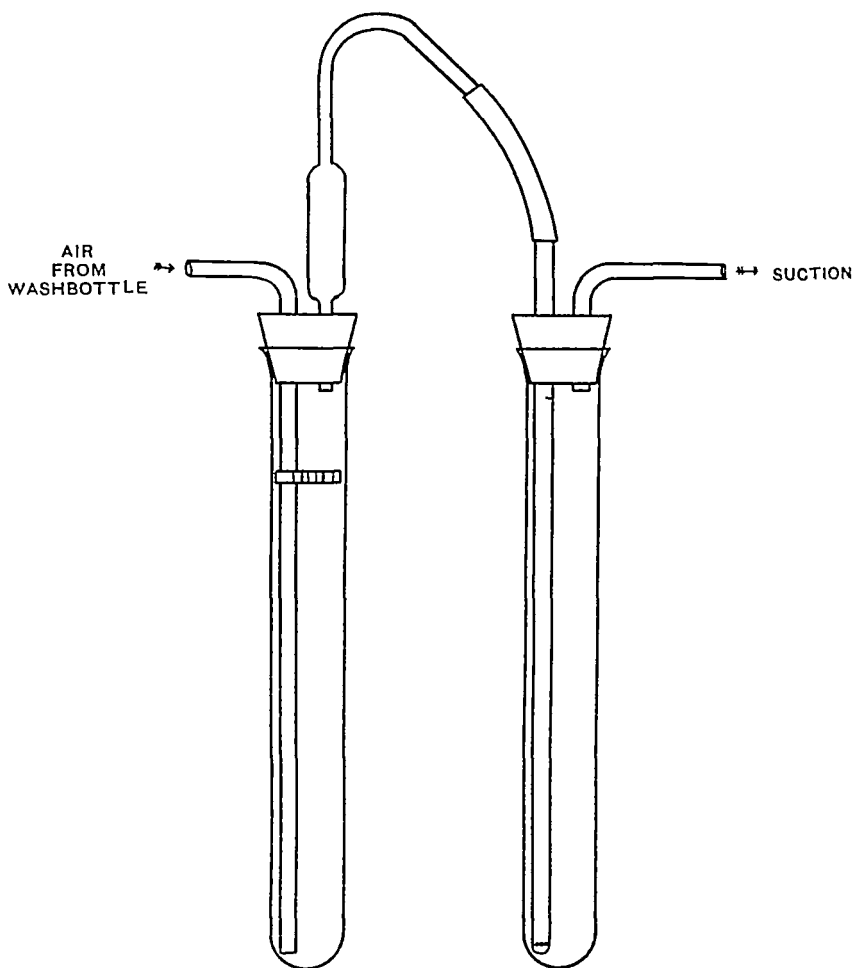


FIG 2 APPARATUS FOR USE WITH SUCTION

obtained is enough to titrate with a very fair degree of accuracy by the help of $\frac{N}{10}$ acid and $\frac{N}{10}$ alkali using alizarin red as indicator. The process is in every way similar to the method described on the preceding pages, except that the ammonia is collected in an ordinary small Florence flask (instead of in a measuring flask or

test tube) containing 10 cc of $\frac{N}{10}$ acid and about 40 cc of water. The solution is titrated in the ordinary manner, and the end point is sufficiently sharp to give very satisfactory results. Results obtained in this way are recorded below. Those who are color blind as well as those whose ability to match colors is rather poor can use the above miniature Kjeldahl process to good advantage.

Had the problem been purely a problem of total nitrogen determinations it is doubtful whether it would have been worth all the time that it has cost to develop the colorimetric procedure after it once had become clear that the color reaction seemingly could not be applied directly to the digestion mixture (see p 493). As will be seen from the other analytical methods now published (see pp 507-536) the total nitrogen determination was only one part of a general colorimetric scheme of analysis.

The determinations recorded below are cited to show the accuracy of our new method for the determination of nitrogen in urine. The middle column represents figures obtained by titrating the ammonia as described above. The figures represent grams of nitrogen per liter of urine.

	NEW METHOD		KJELDAHL'S METHOD	
1	7.9	8.1	8.0	
2	10.0	10.2	9.9	
3	3.7	4.1	3.7	
4	10.5	10.0	10.2	
5	3.8	4.1	3.9	
6	9.4	9.3	9.2	
7	7.5	7.3	7.3	
8	9.2	9.3	9.2	
9	9.0	9.1	9.0	
10	9.3	9.1	9.2	
11	8.5		8.3	
12	9.1		9.3	
13	9.1		9.4	
14	5.2		5.3	
15	3.7		3.7	
16	7.5		7.7	Diabetic urine
17	7.5		7.6	
18	8.4		8.4	Diabetic urine
19	13.1		13.1	Nephritic urine
20	10.0		10.2	Nephritic urine

AN APPARATUS FOR THE ABSORPTION OF FUMES

By OTTO FOLIN AND W. DENIS

(From the Biochemical Laboratory of Harvard Medical School, Boston)

(Received for publication, April 12, 1912)

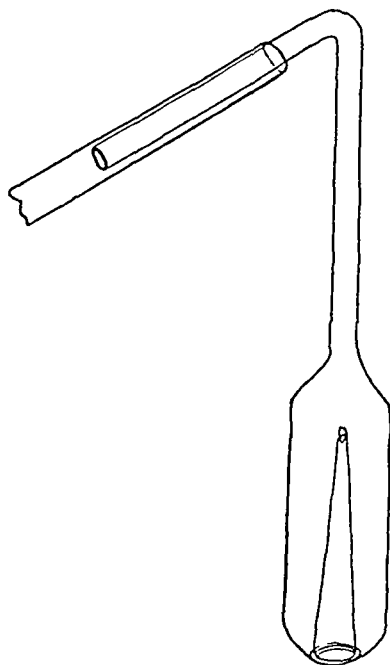
The new microchemical method for the determination of total nitrogen in urine described in the preceding paper requires very little in the way of laboratory equipment except a hood to carry off the sulphuric acid fumes. The decomposition of even a trace of urine with boiling sulphuric acid does produce more irritating fumes than would be tolerated in any small laboratory though the amount is insignificant compared with that produced in an ordinary Kjeldahl digestion.

To overcome this difficulty we have devised an inexpensive little apparatus which has proved surprisingly effective for the removal of such fumes.

As originally made it consisted merely of a broken pipette resting on top of the test tube in which the fumes were generated and drawing off the fumes by means of a water pump (an ordinary cheap one made entirely of glass). For the sake of safety we led the fumes through a large bottle containing a 10 per cent solution of sodic hydrate. We still use this bottle as an accessory although we now know that very little acid comes off and believe that it would probably be perfectly safe to let the fumes run directly through the pump and into the pipes that carry off the water from the latter.

This arrangement had one drawback. There was always more or less condensation of acid and water in the body of the pipette and this acid solution would drain back into the test tube and thus delay the process of decomposition and in addition would drip on the table top when the absorber was removed from the test tube. To overcome this deficiency we sealed up the lower end of the pipette and while still hot and soft invaginated it by pushing the bottom upwards by means of a pointed stick of wood.

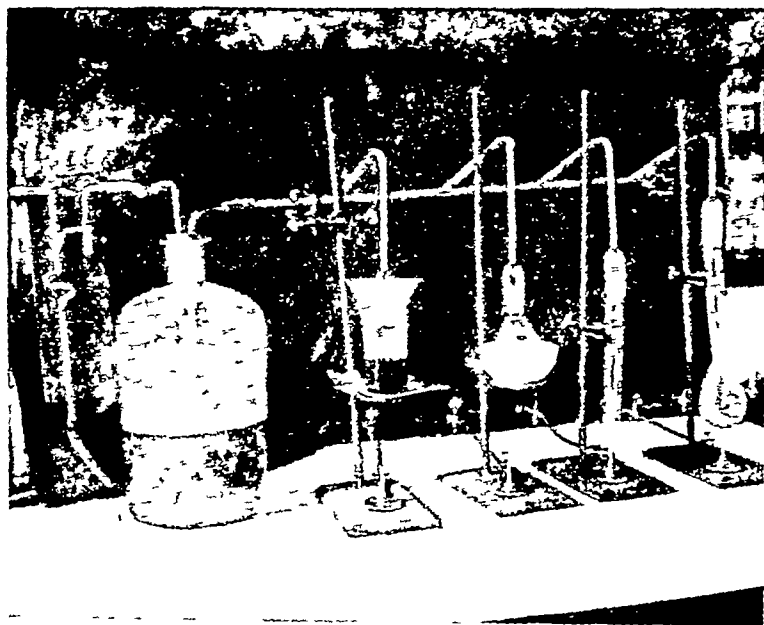
A small hole was then made with a long wire nail in the tip of the invagination and we thus secured a capacious pocket large enough to hold the condensed water and acid obtained from a dozen digestions (see illustration) The fume absorber thus made is



abundantly capable of taking care of all the fumes made in the ordinary Kjeldahl, or Neumann digestion as well, and we now do not use the hood at all for such purposes With the additional help of funnels cut off half an inch above the stem and ground smooth on a wet grindstone, the apparatus, we find, can even be used for carrying off fumes from beakers and evaporating dishes The accompanying photograph shows a somewhat more elaborate apparatus and how it may be used for carrying off fumes from test tubes, flasks, beakers and evaporating dishes

This apparatus is made for four exhaust tubes run by a single pump To accomplish this a large bore glass tube carrying four side tubes connects on the one hand with the pump (or rather with the bottle containing the alkali) and on the other with the individual absorbers The only important point about its construction is that the side tubes shall be of such a diameter that

the bent stems of the absorbers can just slip in for a distance of several inches. The joint thus made, particularly when wet, is quite tight enough and no rubber connection is needed. One or two or all the exhaust bulbs can be used without changing anything and when any one exhaust tube has been used a number



of times and is nearly full of condensed water and acid it is simply withdrawn, emptied and rinsed, and is again ready for use.

We believe that the single absorber at each student's desk might prove a valuable accessory in class room laboratories where hoods so often are inadequate and ineffective.¹

¹ Eimer and Amend now make the apparatus for us and it will be listed in their next catalogue.

ON THE DETERMINATION OF UREA IN URINE

BY OTTO FOLIN

WITH THE ASSISTANCE OF C J V PETTIBONE

(From the Department of Biological Chemistry of Harvard Medical School)

(Received for publication, April 12, 1912)

I	Observations on my magnesium chloride method and on Benedict's method	507
II	A new method in which the urea is decomposed with phosphoric acid	512
III	A new method in which the urea is decomposed in boiling potassium acetate solution	513
IV	(With W DENIS) The determination of urea in the presence of sugar	520

I

The apparent simplicity of the magnesium chloride method for the determination of urea in urine has proved rather deceptive and although this method probably has been used and is still used more frequently than any other in connection with metabolism work the literature which has grown up around it during the past ten years is not lacking in unfavorable criticisms

The chief source of error in the determination is due to incomplete decomposition of the urea. Earlier investigators (Hugounenq, Kossel) had shown that urea is quantitatively decomposed when heated for a short time in sealed tubes or in the autoclave and had published methods for the determination of urea in urine based on that principle. In the magnesium chloride method the boiling point of the mixture containing the urine or urea solutions is raised by the addition of the salt and the rather high temperature needed for the speedy decomposition of the urea is thus secured in a most convenient manner. The essential point in the process is of course that a temperature of not less than 150° shall be maintained in the urea solution for the prescribed period. Lack of experience in how to obtain and to maintain this tem-

perature is the chief cause of the failures to get accurate results. One important factor in this decomposition of urea into ammonia and carbonic acid has, however, not been adequately recognized, namely this, that the time necessary for the complete decomposition of urea under uniform conditions of volume and temperature depends very materially on the amount of urea to be decomposed. That the time of decomposition of urea depends among other factors upon the amount taken is of course an elementary, self-evident proposition but that the small amount of urea represented by the difference between 5 cc of dilute and 5 cc of concentrated urine may require almost a whole extra hour's heating (at 150°C) is anything but self-evident. In this fact is to be found the explanation why the time of heating in the method has been gradually increased from thirty minutes to an hour and a half. It also indicates that the heating time can again be reduced to thirty or forty minutes by limiting the amount of urea taken for an analysis to a maximum of 60 or 70 mgms.

In 1908 Kober¹ called attention to the fact that the long duration of the distillation of the ammonia in the determination of urea is due not to the formation and subsequent decomposition of cyanuric acid derivatives, as I had suggested in my first paper on the subject, but that it is due to the presence of the large amounts of magnesium chloride.

That such is the case I had found a couple of years before the appearance of Kober's paper when I tried to remove the ammonia by distillation from a large batch (several pounds) of magnesium chloride. The hypothesis that condensation products similar to those obtained when urea is heated in a dry condition may be formed was advanced on the basis of the assumption that free water is practically absent from the mixture,² an assumption recently revived by S. R. Benedict³ as explaining why the method is more accurate than the autoclave methods.

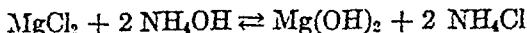
Kober implied that it is practically impossible to distil off ammonia from solutions containing magnesium or calcium salts and by inference that my method for determining urea is hopelessly unsuitable. Without publishing any experiments on the subject

¹ *Journ Amer Chem Soc*, xxx, p 1279, 1908

² *Zeitschr f physiol Chem*, xxxvi, p 336, 1902

³ *This Journal*, viii, p 415, 1910

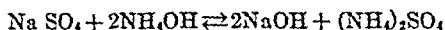
Kober leaves it to be understood as "quite obvious" from the mass law and the reversible reaction



that the ammonia cannot be obtained by distillations from solutions containing 15 to 20 grams of calcium or magnesium salts as in my method for determining urea

In a recent article on the determination of urea Henriques⁴ and Gammeltoft have reproduced Kober's deductive argument against my method in a rather more specific and positive form. Experiments on the subject are, however, still missing.

But the mass law is only generalization. The reversible reaction quoted above conveys no information as to the final outcome of the distillation of ammonia in the presence of magnesium salts. The analogous reaction



can equally well be presented for the ammonia distillation in the Kjeldahl process yet we know from experience that distillation yields satisfactory results.

The question how completely ammonia can be obtained from ammonium salts under the conditions of distillation prevailing in the urea determination is of course easily determined experimentally. The results cited below were obtained in November 1908 in the order given from a standard ammonium sulphate solution (25 cc of which contained 25.5 cc $\frac{N}{10}$ NH_3) when distilled for one hour with 15 grams of magnesium chloride,⁵ 700 to 800 cc water and 20 cc 7.5 per cent sodium hydrate solution.

(1) 25.5	(7) 25.4	(12) 25.5	(18) 25.45
(2) 25.5	(8) 25.4	(13) 25.3	(19) 25.35
(3) 25.25	(9) 25.5	(14) 25.4	(20) 25.4
(4) 25.25	(10) 25.5	(15) 25.6	(21) 25.35
(5) 25.5	(11) 25.5	(16) 25.5	(22) 25.4
(6) 25.5	(12) 25.3	(17) 25.5	(23) 25.4

When the amount of ammonia in the solution was doubled so that it contained 51 cc $\frac{N}{10}$ NH_3 the results of the distillation

⁴ *Stand. Arch. f. Physiol.*, xxv, p. 154, 1911.

⁵ The magnesium chloride used was free from ammonia and as it was a fused salt (in sticks) 15 grams were used instead of 20 grams for these distillations just as in the urea determinations.

were less satisfactory After one hour's distillation the following figures were obtained

(1) 50 3	(5) 49 9	(9) 50 1
(2) 50 3	(6) 49 8	(10) 50 25
(3) 50 0	(7) 49 9	(11) 49 9
(4) 50 3	(8) 50 0	

When distilling according to the directions given for urea determinations, *i e*, until the distillates failed to give an alkaline reaction with litmus paper, the figures cited below were obtained (in about one hour and twenty minutes)

(1) 50 8	(3) 50 65	(5) 50 65	(7) 50 85
(2) 50 7	(4) 50 5	(6) 50 6	(8) 50 7

The above results are in harmony with the fact that so many different investigators not only in my laboratory but in many other laboratories have obtained satisfactory results for the nitrogen of pure urea solutions The difficulties in so far as there have been any have come when urines of various concentrations were substituted for the urea solutions and the chief cause of these difficulties as mentioned above has been the incomplete decomposition of the urea

The chief criticisms raised against the magnesium chloride method for the determination of urea is not that it is less accurate than any other method but that too much skill, experience and time is necessary in order to obtain reliable results ⁶

In his last paper on the estimation of urea S R Benedict (*loc cit*) describes a new method which he believes to be very accurate, giving figures slightly lower than those obtained by means of the magnesium chloride method, though "the agreement between the two methods is often as close as two duplicate determinations by the same method"

In working on pure products, creatinine, uric acid, and allantoin, Benedict finds that whatever difference there is between the two methods is rather in favor of his new one He clearly recognizes, however, that the ammonia obtained from those pro-

⁶ It is interesting to note that this criticism comes chiefly from American laboratories where metabolism experiments for the past few years have been conducted on a wholesale, factory-like basis

ducts in urine work is negligible and accordingly recommends the use of sodium hydroxide instead of sodium carbonate for distilling off the ammonia. As a matter of fact the method which he thus recommends would yield according to his own experiments fully as much ammonia from creatinine + uric acid as does the "Folin" method, and he did not try this method with allantoin. As the method he did try with allantoin decomposed more than 50 per cent of 30 mgms it is reasonably certain that it would decompose quantitatively such small traces of allantoin as may be present in 5 cc of urine.

The following urea determinations in urines made by Benedict's method (using sodium carbonate as alkali) and by the magnesium chloride method show that the two do indeed yield substantially the same results. The figures represent grams of nitrogen per liter of urine.

FOLIN'S METHOD	BENEDICT'S METHOD	NEW PHOSPHORIC ACID METHOD*
5.1	5.2	5.2
6.6	6.6	6.5
2.9	2.8	2.8
2.7	2.7	2.7
3.3	3.4	3.3
14.4	14.2	14.1
5.9	5.8	5.7
17.5	17.4	17.4
12.8	12.8	12.7
6.5	6.5	
2.0	2.0	2.0
8.8	8.7	8.7
8.6	8.5	8.6
7.4	7.2	7.3
8.9	8.8	8.8
9.5	9.4	9.4

See p. 512

The magnesium chloride method as used for the above determinations has been somewhat simplified in that the decomposition is carried on in a Kjeldahl flask (capacity 500 cc) by the help of a small so-called micro-burner. A large test tube filled with cold water and suspended in the neck of the flask by means of a cork or copper wire is used as a condenser. Only one-half of

the test tube should be inside the Kjeldahl flask. After adding about 350 cc water (hot) and alkali the ammonia can be distilled off directly from the Kjeldahl flask in about an hour notwithstanding the higher initial concentration of the magnesium chloride.

II

On p 508 I indicated that since the decomposition time depends in a large measure on the actual amount of urea to be decomposed that time could be very materially reduced by diluting the urine so that not over 60 or 75 mgms of urea is used for each determination. At best, however, the determination would probably require over two hours by the magnesium chloride method. The task which I have endeavored to accomplish was to evolve a method for the determination of urea which should at least approximate in speed and convenience the method for total nitrogen described in the preceding paper.

The problem was to decompose one or a few milligrams of urea and either titrate the ammonia with very dilute acid and alkali or to determine it colorimetrically by means of Nessler's solution.

The magnesium chloride was found not to be suitable as a means of producing the necessary temperature on such a small scale. The procedure described below accomplishes the purpose fairly well with 1 cc of undiluted urine.

Measure the urine (1 cc) with an Ostwald pipette into a Jena test tube. Add three good sized drops of pure phosphoric acid, one drop of indicator (alizarin red), a few grains of talcum powder and boil the mixture over a free flame until about one-half of the water has escaped. This requires only two to three minutes. Now place the test tube in a bath (paraffin, oil, or sulphuric acid), previously heated to 175° to 180°C for fifteen minutes. The urea is completely decomposed in that time. The content of the tube is then dissolved by the addition of water (1 to 2 cc) and a little heat. After adding 0.5 to 1 cc of 50 per cent caustic potash⁷ the ammonia is removed by a strong air current in ten minutes. It is collected in 25 cc of $\frac{N}{10}$ hydrochloric acid and the excess of the acid is titrated with $\frac{N}{10}$ sodium hydroxide using

⁷ KOH is better than NaOH because of the greater solubility of potassium phosphate.

alizarin red as indicator With the paraffin bath in order this determination can be finished in about half an hour Results obtained in this manner and calculated in grams per liter are cited on p 511 No one would hesitate to consider those figures satisfactory They were obtained by Mr Pettibone only after several months' fruitless endeavor in other directions

III

The method just described while representing a great saving of time when compared with any other reliable method was not considered entirely satisfactory Like Benedict's new method it depends on a bath, kept at a certain temperature, for the heat that is to decompose the urea While this may not be much of a drawback, particularly if one has to make a large number of determinations at the same time, still it is a drawback that it seemed worth while to endeavor to get rid of To solve the problem I have returned to the principle used in the magnesium chloride method, *ie*, the use of a salt to obtain the high boiling point necessary for the speedy decomposition of urea

The salt finally adopted for this purpose is potassium acetate By means of this salt any temperature up to 158° to 160° can be obtained Potassium acetate is unfortunately somewhat hygroscopic though less so than magnesium chloride The hygroscopic quality is, however, more objectionable in the new method about to be described because one of the advantages striven for is to get around the preliminary boiling off of water called for in the phosphoric acid method just described as well as in my earlier magnesium chloride method and in Benedict's method With any dry salt and a definite amount of water any given temperature which that salt is capable of giving might be obtained at once without any preliminary concentration provided enough of the salt is taken Being rather hygroscopic, the different brands of potassium acetate on the market differ markedly in the amount of water they contain The best German brands are sufficiently dry for the purpose here involved while the American brands, as at present sold, contain very much more water and should be dried before being used The salt loses its water very readily and we dry it, about a pound at a time by having it in a large

porcelain dish standing on a warm plate (at about 115°) for about twenty-four hours. The plate must not be too hot as the acetate decomposes rather easily. The method described below is based on the use of such dry salt.

An important accessory in this new procedure for the determination of urea is a temperature indicator. This indicator was originally devised for use in connection with the magnesium chloride method but it has proved less useful there than in connection with the new method because in the old method so much coloring matter is formed as to obscure the indicator. This temperature indicator consists of powdered chloride-iodide of mercury (HgI_2) inclosed in a sealed glass bulb not over 1 mm in diameter. This salt is bright red at ordinary temperatures. At 118°C it turns lemon yellow and melts to a clear dark red liquid at 155°C . It solidifies again at about 148°C and resumes its red color gradually only in the course of about twenty-four hours. The melting point temperature 153°C is fortunately a temperature very readily obtained and maintained by means of potassium acetate and as the acetate begins to cake and solidify at 160° to 161° there is no danger in this combination of having either too high or too low a temperature without its being unmistakably apparent.

The salt in question, HgI_2 , is prepared by heating in a dry state intimately mixed mercuric chloride and mercuric iodide in molecular proportions at 150° to 160°C for six to eight hours. At the end of the heating the product should be powdered and used as it is for it cannot be purified by the use of solvents. It should be kept dry until sealed up as indicated.^s

Since the urea according to this method is decomposed in a practically saturated potassium acetate solution, the acid to be used for retaining the liberated ammonia must of course be acetic acid. Acetic acid in the presence of so much acetate is an extremely weak acid. In fact it is barely capable of holding the ammonia under the conditions of the determination so that for a time it was thought that the low results which were constantly obtained were due to the escape of ammonia. The decompo-

^s Kohler *Ber d d chem Gesellsch*, 11, p 1187, 1879. The indicator properly sealed up in bulbs as well as the other special appliances needed in this determination can, however, be obtained from Eimer and Amend, New York.

sition of the urea in this method may therefore be said to be accomplished in an almost neutral medium. As indicated by alizarin red, the medium is neutral or alkaline, certainly not acid.

The method is as follows.

The urine is diluted so that 1 cc contains 0.75 to 1.5 mgms of urea nitrogen. Dilutions of 1 in 20, 1 in 10 or rarely 1 in 5 are usually adequate for this purpose. One cubic centimeter of the diluted urine is then transferred by means of an Ostwald pipette to a large Jena test tube (200 mm by 20 mm) previously charged with 7 grams of dry potassium acetate (*free from lumps*), 1 cc of 50 per cent acetic acid, a small sand pebble, or better, a little powdered zinc (not zinc dust) to prevent bumping during the boiling, and a temperature indicator.

The test tube is then closed by means of a rubber stopper carrying an empty narrow "calcium chloride tube" (without bulb) as a condenser (size of calcium chloride tube, 25 cm by 1.5 cm). The test tube and condenser are then suspended by a burette clamp or similar device so that it can easily be raised or lowered with reference to the small flame of the micro-burner. As soon as the acetate is dissolved and the mixture begins to boil, which usually occurs in about two minutes, the indicator begins to melt showing that the desired temperature (153° to 160°C) has been reached. The boiling is continued in a gentle, even manner for ten minutes at the end of which time the decomposition of the urea is already completed. The apparatus is removed from the flame and the contents are diluted by the addition of 5 cc of water. The water is added by means of a pipette through the calcium chloride tube so as to rinse the sides of the tube and the bottom of the rubber stopper from traces of ammonium acetate which may be there. Not more than 5 cc of water should be used for this purpose. An excess of alkali, 2 cc of saturated sodium hydrate or potassium carbonate solution, is added and the liberated ammonia is driven off by means of a strong air current into a 100 cc measuring flask containing about 35 cc of water and about 2 cc of $\frac{1}{10}$ acid. The time required for this will of course depend on the strength of the air current. In this laboratory ten minutes is allowed and is abundant. The ammonia thus set free is determined colorimetrically against 1 mgm of nitrogen in the form of ammonium sulphate exactly as in the total nitrogen determination described in the preceding paper.

In execution the determination of urea described above is about as simple and free from complications requiring unusual skill or experience as it is possible to make a quantitative method. While in the process of development, however, this was not the case and it sometimes appeared as though it would not be possible to find the conditions which could be depended on to yield theoretical results.

For a long time the results were almost invariably too low although an occasional theoretical figure showed that such was not necessarily the case. The deficiency in the ammonia found was supposed to be due to the inability of acetic acid to prevent its escape and numerous futile efforts were made to detect the loss and to prevent it. In time the losses were found to be due to the formation of condensation products which do not give up their ammonia to the air current and it was further found that the acetic acid concentration or the absence of water was the factor which determined this formation. Because of the weakness of acetic acid in concentrated acetate solution, glacial acetic acid rather than dilute acid was used to retain the ammonia. This was wrong.

With glacial acetic acid and dry acetate, whether two or three drops or any larger quantity is used, the results were almost invariably too low. And the greater the amount of acid taken the greater was the loss of nitrogen. This fact suggested that probably acetamide was formed. But when ammonium sulphate was substituted for urea there was no loss. Later it was found that when 1 cc of ammonium sulphate solution containing 5 mgms of nitrogen or over was used with glacial acetic acid, all of the ammonia could not be recovered by means of the air current, though it could be obtained by distillation, thus showing that acetamide was probably formed. But since urea behaved similarly when only 1 mgm of urea nitrogen was present it was clear that the amide formation could not be the cause of the failure to recover it all. The acetamide theory furnished, however, the solution of the problem from the analytical standpoint. By substituting 50 per cent acetic acid for the anhydrous acid the difficulty disappeared. Urea corresponding to as much as 5 mgms of urea nitrogen will be completely decomposed by ten minutes' boiling with 7 grams of potassium acetate and 1 cc of 50 per cent acetic

acid and the ammonia will be recovered quantitatively by means of the air current. Five or six milligrams of nitrogen represents, however, the upper limit under the conditions described. If 10 mgms of nitrogen are taken, whether in the form of urea or of ammonium sulphate, 50 per cent acetic acid does not entirely prevent the formation of more or less stable condensation products. When as much as 10 mgms of ammonia are present there is also danger of losing some mechanically for in the upper half of the test tube there is then an abundance of ammonia as well as of acetic acid vapors during the boiling. It is of course desirable that this ammonia be kept down as near the boiling liquid as is practicable. Consequently it is desirable first to avoid bumping and secondly to keep the steam pretty well confined.

To keep the steam down the amount of water present in the system must be kept low. It is possible to get a temperature of 153°C and over with only 3 grams of potassium acetate and 2 cc of water by boiling the mixture so hard that the surplus water is constantly kept circulating in the upper part of the test tube. The whole test tube and the lower half of the condenser as well, will then be very hot from contact with the steam. A similar result is of course obtained by using 7 grams or even more of acetate which is not dry. By using 7 grams of reasonably dry acetate, however, one obtains with 2 cc of water a mixture which can be gently boiled above 153°C with the evolution of so little steam that the upper part of the test tube remains quite cool. The flame from the micro-burner necessary to maintain boiling in such a solution need not be over 0.5 cc long and, at that, the bottom of the test tube must be some distance above it.⁹ If too much heat is applied the acetate cakes at the bottom of the mixture, if too little it cakes at the top. With the small flame from a micro-burner and a windshield it is, however, very easy to keep solution boiling without caking.¹⁰

A few additional points should be mentioned in connection with this new method for determining urea.

⁹ Bottomless beakers make excellent windshields for such small flames and wind-shields of some sort are indispensable in most laboratories.

¹⁰ Such a boiling solution was once left over night and was found in the same condition, i.e., boiling and clear, in the morning.

1 When the urea is decomposed in boiling acetate solution at 150° to 160° that solution as already indicated is only faintly acid. The solution does not retain quantitatively either acetic acid or ammonia and a certain amount of each is present in the vapors above the boiling mixture. In a pure water solution of ammonia and acetic acid, on the other hand, the ammonia does not escape with the vapors when boiled provided the amount of ammonia present is not too large. The acetic acid vapors above the acetate solution therefore probably help very much to keep the ammonia from escaping.

2 Bumping in a boiling test tube is always disagreeable. In this case no bumping whatever is wanted, first because the vapors inside are charged with more or less ammonia and secondly because in a bumping solution the acetate will suddenly cake at the bottom. Bumping is easily prevented by the presence of a rough piece of gravel the size of an ordinary glass bead. A small pinch of powdered zinc is even better than the pebble for this purpose. The acidity of the solution is so weak that the action on the zinc in spite of the high temperature is very slight.

3 Curiously enough the presence of the zinc appears to somewhat modify the hydrolytic power of the hot acetate mixture. It reduces to a marked degree the decomposition of allantoin yet does not interfere with the decomposition of urea. In the presence of zinc not over one half of half a milligram of allantoin-N can be recovered.

4 The essentially neutral acetate mixture used in this method represents probably the mildest direct hydrolysis yet applied for the purpose of determining urea in urine. Other urinary constituents, except of course the ammonia, contribute probably very little indeed to the result. Neither creatinine nor hippuric acid gives even a trace of ammonia. Uric acid sometimes seems to give enough to make the qualitative test positive, at other times the qualitative test is negative and, at all events, the test (whether much or little uric acid is taken) is quantitatively imperceptible when made in the presence of standard urea solutions (see next page). Allantoin, as already indicated, may give off about one half of its nitrogen in the presence of zinc, otherwise it behaves like urea provided its quantity does not exceed 0.5 mgms of allantoin-N. The darkening of urine, conspicuous in the mag-

nesium chloride method, is almost entirely absent in the acetate mixture even when undiluted urine is subjected to the treatment. In this respect the marked charring effects obtained in the phosphoric acid method described above, as well as in Benedict's acid sulphate method, is rather disconcerting, though apparently harmless ¹¹

5 As with the total nitrogen determination described in the preceding paper the best air current is compressed air since the ammonia can then most conveniently be collected directly in the 100 cc measuring flask. Suction with a good water pump can be used, however, and as we have satisfied ourselves repeatedly, will also take out all the ammonia in ten minutes. The most rapid stream of which the pump is capable should always be used for there is no danger of losing any ammonia (see p 523)

6 For preparation of the standard ammonium sulphate solution, the Nessler solution, and for the details of the color comparison, etc, see the preceding paper

7 This method has been designed primarily for a colorimetric reading of the ammonia. As the method is perfectly reliable for larger quantities of urea up to 5 mgms of nitrogen titrations with $\frac{N}{50}$ solutions may be applied. With a colorimeter at hand the colorimetric method is, however, equally convenient and rather more accurate

8 The test tubes in which the urea is to be decomposed should be dry. The amount of water present in a freshly rinsed test tube is considerable, relative to the total amount present in the reagents

The parallel urea determinations recorded below were made for the purpose of determining whether this new method gives essentially the same values as the magnesium chloride method. I expected rather lower results with the new method but this

¹¹ The home dried potassium acetate used in these determinations was as a matter of fact not entirely free from ammonia when tested qualitatively for it. The trace found was sometimes a trifle increased, sometimes not, after uric acid had been heated in the mixture. In no case, however, did these traces appreciably affect the color of the ammonia corresponding to 1 mgm of nitrogen. Potassium acetate containing less than 1 per cent of moisture and free from ammonia is now made for us by J T Baker Chemical Company, Phillipsburg, N J

expectation was not realized. The differences are immaterial. The figures represent grams of urea-N per liter of urine.

COLORIMETRIC METHOD	MAGNESIUM CHLORIDE METHOD
14.2	14.1
8.2	8.2
8.4	8.5
6.7	6.8
10.5	10.6
8.6	8.7
3.3	3.4

IV

The determination of urea in urines containing sugar has been recognized as a special problem ever since the publication in 1903 of Morner's illuminating paper on the different methods then available for the determination of urea in human urine. Morner's own procedure¹² for the preliminary removal of the sugar has remained a tedious but indispensable prerequisite for the determination of urea in such urines. None of the methods published since that time represent any improvement in this respect and the determination of urea in diabetic urines is still a comparatively long and laborious operation. The colorimetric potassium acetate method described above appeared at first to be no more suitable for sugar urines than any other. In the presence of sugar the results obtained were invariably from 20 to 50 per cent too low. A more systematic investigation of the subject has, however, shown that it is possible by means of this method to meet the unusual conditions which must be fulfilled if urea is to be quantitatively converted into ammonia in the presence of sugar.

The reason why sugars interfere with the decomposition of urea was formerly ascribed to the formation of nitrogenous "melanins" but the loss of nitrogen is in all probability due to the formation of definite, stable ureids.¹³ The difficulty involved is therefore analogous to the difficulty encountered in the use of acetic acid (see p. 516). The disturbing effects of acetic acid were overcome

¹² Morner *Skand Arch f Physiol*, xiv, p. 319

¹³ Folin *Amer Journ of Physiol*, xiii, p. 46, 1905

by reducing its concentration below the point at which it begins to give condensation products with urea. The remedy against the ureid-forming tendency of the sugars is the same. When the amount of sugar present is sufficiently small the combination with urea does not take place and the results obtained are quantitative.

The dilution necessary to prevent the fatal ureid formation in the case of dextrose is, however, very great, so great in fact, as to be entirely out of reach in the titration methods for determining urea.

The presence of 10 mgms of dextrose in the acetate mixture used to decompose urea (about 2 mgms) is accompanied by a loss of 40 to 50 per cent of urea nitrogen. With 5 mgms of dextrose present the loss of urea nitrogen sinks to about 20 per cent and this loss remains about the same whether the urea nitrogen present is 1 mgm or is reduced to one half or even to one-tenth of that amount.

The losses due to sugar depend therefore chiefly on the amount of sugar present and only to a much smaller extent upon the amount of urea to be decomposed. One milligram of urea-nitrogen is, however, too large an amount for a determination in the presence of sugar. With this amount a loss of about 5 per cent is encountered in the presence of 1 mgm of dextrose.

With the colorimetric method for determining ammonia, a method which until now has been used only in water analysis, it is of course possible to determine much smaller amounts of urea than those corresponding to 1 mgm of nitrogen. In fact it was only by virtue of the special adaptation of the method worked out in this laboratory that it became possible to work with as much as 1 mgm or more of nitrogen. One tenth of a milligram of nitrogen can be determined with a very satisfactory degree of accuracy by diluting the Nesslerized ammonia to only 10 cc (instead of 100 cc) before reading the color. But experiments have shown that 0.1 to 0.3 mgm of urea nitrogen can be determined in the presence of as much as 2 mgms of dextrose. It is therefore possible by simply diluting diabetic urine until 1 cc contains about 0.1 mg of urea nitrogen to determine the urea without any preliminary removal of the sugar when the dextrose-nitrogen ratio (D/N) is as high as 20:1.

The determination is made as follows: 1 cc of urine previously

diluted from 20 to 100 times is decomposed in the usual manner with the potassium acetate and acetic acid. The ammonia is then driven into a second test tube containing about 2 cc of water and 0.5 cc of $\frac{N}{10}$ hydrochloric acid. To the contents of this test tube are then added first, a couple of cubic centimeters of water, then 3 cc of diluted (1.5) Nessler's solution. The colored solution obtained is then rinsed and washed into a 10 cc measuring flask and the volume made up to the 10 cc mark. The whole is transferred to a dry cylinder of a Duboscq colorimeter and the depth of the color is determined in the usual way against the standard containing 1 mg of nitrogen per 100 cc of solution.

The following determinations may be cited to show the extent to which the figures for the urea come up with the dilution of urines containing sugar.

	VOLUME OF URINE	DILUTION	UREA NITROGEN	TOTAL NITROGEN	SUGAR
	cc		grams	grams	per cent
(1)	4000	1 2	7 6	14 2	4 5
		1 20	12 3	14 2	4 5
(2)	4400	1 2	6 9	13 0	4 5
		1 20	11 0	13 0	4 5
(3)	3800	1 2	5 7	11 0	3 7
		1 20	9 5	11 0	3 7
(4)	3800	1 2	6 7	11 4	3 8
		1 20	9 5	11 4	3 8

The following figures were obtained after adding 10 per cent dextrose to normal urines of known urea content.

	DILUTION	UREA NITROGEN FOUND	TRUE UREA NITROGEN PER 100 CC
(1)	1 10	0 62	0 97
	1 100	0 98	0 97
(2)	1 10	0 45	0 69
	1 100	0 69	0 69
(3)	1 10	0 32	0 50
	1 50	0 50	0 50

ON THE DETERMINATION OF AMMONIA IN URINE

BY OTTO FOLIN AND A. B. MACALLUM

(From the Biochemical Laboratory of Harvard Medical School)

(Received for publication, April 12, 1912)

In Folin's air current method for the determination of ammonia 20 or 25 cc of urine is used and from this volume of liquid all the ammonia can be removed in from one to three or four hours, the time depending on the rapidity of the air current. The accuracy of that method has never been questioned. But a number of investigators have abandoned the attempt to make use of it because they did not have a strong enough air current to work with and others, as indicated above, have had to run their air currents several hours in order to obtain all the ammonia when an hour to an hour and a half should be enough. They have assumed that the water pressure in their laboratories has not been sufficient to produce the required air current. This is a mistake. A pressure of 40 to 45 pounds per square inch is probably available in most laboratories and such a pressure is sufficient to produce a very effective air current provided the water pump used is a good one.¹

In the paper on the air current method for determining ammonia attention was called to the fact that the rapidity with which a given air current removes ammonia from solutions depends very much on the volume of the solution. To shorten the time of the determination of ammonia in urine it is therefore only necessary to reduce the volume of urine used. In the two preceding papers

¹ The water pump listed in the Kny-Scheerer Company's Catalogue (List 120), No 2458, p 272, produces, when properly adjusted, an entirely adequate air current with such a water pressure. Its only drawback is that its attachment nut does not fit any American made thread and its attachment therefore requires the help of a mechanic. It should not be bought without the vacuum gauge because the latter makes the adjustment to the point of maximum efficiency very much easier.

(on the determinations of total nitrogen and of urea) it was shown that ammonia could be removed quantitatively from 10 cc of solution in ten minutes or less

The application of the technique described in those papers to the determination of ammonia is more or less self-evident² The ammonia determination according to this method is carried out as follows

Into a test tube measure by means of Ostwald pipettes 1 to 5 cc of urine (The volume taken should give 0.75 to 1.5 mgms ammonia-nitrogen With normal urines 2 cc will most often give the desired amount With very dilute urines 5 cc may be required, while with diabetic urines rich in ammonium salts even 1 cc may give too much and the urine must be diluted) Add to the urine a few drops of a solution containing 10 per cent of potassium carbonate and 15 per cent of potassium oxalate, and a few drops of kerosene or heavy, crude machine oil (to prevent foaming) Pass the strong air current through the mixture for ten minutes (or as long as is necessary to drive off all the ammonia) and collect the ammonia in a 100 cc measuring flask containing about 20 cc of water and 2 cc of $\frac{N}{10}$ acid Nesslerize as described in the paper on the total nitrogen determination, p 495, and compare with 1 mgm of nitrogen obtained from a standard ammonium sulphate solution and similarly Nesslerized

Results obtained by this rapid method and parallel results obtained by the original air current method are given in the table on the opposite page

It is clear that the figures obtained by the new rapid process are practically identical with the figures obtained by the old air current method

No absolutely sharp end-point is obtainable when a rapid air current is passed through urine A trace of something capable of giving a color with Nessler's solutions continues to come long after all the ammonia has been removed This is a point of distinction between urine and ammonium salt solutions What this

² The determination of ammonia as described in this paper is not so far as the development is concerned a mere application of the principles described in the preceding papers On the contrary the investigation of this method was started simultaneously with the other two and was finished first

Grams ammonia-nitrogen per liter of urine

FOLIN'S METHOD	COLORIMETRIC METHOD	FOLIN'S METHOD	COLORIMETRIC METHOD
0 55	0 58	0 80	0 80
0 52	0 54	0 60	0 60
0 44	0 50	0 62	0 62
0 45	0 48	0 26	0 27
0 40	0 45	1 35	1 38
0 43	0 44	0 51	0 54
0 47	0 47	0 44	0 48
0 42	0 42	1 10	1 09
0 37	0 39	1 99	2 00
0 48	0 50		

substance is we do not know, though we have devoted considerable time to its investigation. The effect of this substance in actual ammonia determinations is so small as to be hardly, if at all, perceptible.

NEW METHODS FOR THE DETERMINATION OF TOTAL NON-PROTEIN NITROGEN, UREA AND AMMONIA IN BLOOD

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(Received for publication, April 12, 1912)

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IV Determination of the urea	531
V Determination of the ammonia	532

The analytical technique described in the preceding three papers lends itself peculiarly well to the determination of total uncoagulable nitrogen, urea and ammonia in blood, milk, eggs and other liquids where we are dealing with minute amounts of these different constituents. In two earlier papers results obtained by adaptations of these methods to blood analysis were published.¹ The procedures by means of which these results were obtained are described in this paper.

I METHOD FOR DRAWING BLOOD

Before going into the details of the chemical work it would seem worth while to describe our method of drawing blood because so far as we have been able to learn it is somewhat different from the procedures employed by physiologists and because we believe it to be expeditious, neat and exact and therefore particularly suitable for quantitative work.

We use neither cannulae nor syringes but simply hypodermic needles and pipettes. The needles are about 1 mm in diameter and about 25 mm long. They are immersed in a dilute solution

¹ This *Journal* xi, p. 87, *Ibid*, p. 161, 1912

of vaseline in ether and then allowed to drain and dry on a clean paper for a few minutes before being used (This does not apply of course to the drawing of human blood when the needles must be thoroughly sterilized) An adequate supply of these needles is kept on hand so that we do not need to use any needle more than once in any one experiment The needle is attached to the tip of a 2 or 5 cc pipette by means of a short piece of narrow pure gum tubing A small pinch of powdered potassium oxalate is introduced into the upper end of the pipette (which must be clean and perfectly dry) and is allowed to run down into the tip and the needle The other end of the pipette is connected with a rubber tube which in turn connects with a mouth piece consisting of a short tapering glass tube Close to the pipette the rubber tube carries a pinchcock

To draw the blood one of us inserts the needle in the vein or artery and the other regulates the flow of the blood by means of the pinchcock and by suction The exact quantity of blood desired is thus obtained without any waste and without clotting

II ISOLATION OF NON-PROTEIN NITROGENOUS CONSTITUENTS

To separate the non-protein nitrogenous constituents from the protein materials we make use of pure (acetone-free) methyl alcohol and an alcoholic solution of zinc chloride Ordinary methyl alcohol cannot be used because the impurities in it, particularly the acetone, combine with more or less of the urea so that it escapes decomposition in the subsequent treatment and is not quantitatively recovered We have satisfied ourselves by means of determinations on pure urea solutions that the presence of acetone results in a loss of urea

As soon as the blood is drawn it is transferred into measuring flasks half filled with methyl alcohol and the flasks are then filled up to the mark with methyl alcohol and vigorously shaken Two cubic centimeters of blood we dilute to 25, while for 5 of blood we use 50 cc flasks At the end of two hours, or as soon after that as is convenient, the contents of the flasks are filtered through dry filters To the filtrate are then added two or three drops of a saturated alcoholic solution of zinc chloride and after standing for a few minutes the mixture is again filtered through a dry

paper The zinc chloride brings down an appreciable precipitate and the last traces of coloring matters so that when the second filtration is made a perfectly colorless filtrate is obtained Five cubic centimeters of these filtrates, corresponding to 0.4 or to 0.5 cc of blood, depending on whether 2 or 5 cc of blood were drawn, are taken for each determination The precipitation procedure described above is the one which we ordinarily use There are objections to it We are not certain that traces of protein-like materials may not escape precipitation by this as by every other method and we do know that the filtrate does not contain all of the non-protein materials When relatively large quantities (equivalent to 100 mgm of nitrogen per 100 cc of blood) of creatine, or asparagine are added to blood and treated as described above there is invariably an appreciable loss of material To overcome this loss we have tried to triturate and wash the first alcoholic precipitate with methyl alcohol, and with some substances, as for example, with glycocholic acid, urea and acetamide, we are thus able to get practically quantitative results while with others, such as creatine, asparagine, and tyrosine, we still do not get quite all Moreover, such trituration and washing does leach out a small amount of the coloring matters of the blood so that except for special experiments with less soluble substances we consider the simpler procedure rather more satisfactory

In the case of muscle analysis, on the other hand, we thoroughly triturate and wash with the alcohol Incidentally it should be said that, muscles as soon as cut out, while still twitching, are cut with a pair of sharp scissors and immediately immersed in methyl alcohol (about 50 cc in an Erlenmeyer flask) After being allowed to stand for a few hours the coagulated muscle is thoroughly ground up and then extracted overnight with a fresh portion of alcohol The various extracts and washings are then combined, filtered into a 100 cc volumetric flask and after the addition of a few drops of alcoholic zinc chloride solution, made up to volume with methyl alcohol and again filtered We invariably start with 5 grams of muscle and use 10 cc of the filtrate for each determination of total nitrogen as well as of urea

III DETERMINATION OF THE TOTAL NON-PROTEIN NITROGEN

To determine the total non-protein nitrogen of the blood 5 cc of the alcoholic filtrate is transferred to a large Jena test tube of the same kind as is used in urine analysis (see p 494) One drop

of sulphuric acid, one of kerosene and a pebble are added and the methyl alcohol is driven off by immersing the test tube in a beaker of boiling water for five to ten minutes. When the alcohol is removed 1 cc of concentrated sulphuric acid, a gram of potassium sulphate, and a drop of copper sulphate solution are added and the mixture is boiled, cooled and diluted as in the analysis of urine (see p 494)

From this digestion mixture the ammonia is removed in the usual manner. It is, however, not collected directly in a measuring flask (as in urine analysis) but in a second large test tube previously charged with 1 cc of $\frac{N}{10}$ acid and 2 to 3 cc of water. The reason for this variation is that 0.4 to 0.5 cc of blood contains only 0.1 to 0.2 mgm of non-protein nitrogen. The final Nesslerized solution cannot be diluted to 100 cc and smaller volumetric flasks cannot be used as receivers during the air current treatment because of spattering. Large test tubes are therefore used as receivers and the ammonia is Nesslerized in these before the liquids are transferred to measuring flasks. Ordinarily the colored solutions obtained from cat's blood are transferred to 25 cc flasks and are then found to have a depth of color which permits of a sure and accurate reading in the colorimeter. In some of our absorption experiments the total non-protein nitrogen runs up to very high figures and then the solutions are diluted to 50, sometimes even to 100 cc, before being read in the colorimeter.

Human blood contains scarcely more than one half as much non-protein nitrogen as cat's blood. In the case of human blood we therefore never draw less than 5 cc and we take 10 cc of the filtrate for each determination. In all other respects we use the same procedure for human blood as for cat's blood. In all ordinary cases 7 to 8 cc of diluted Nessler's reagent (dilution 1:5) are added for the production of the color. If much ammonia is present so that the resulting colored solution must be diluted to 50 or 100 cc correspondingly larger amounts of Nessler's reagent are added.

The calculation of the analytical results to milligrams of nitrogen per 100 cc of blood is not difficult but the formulae given below may prove useful. In these formulae the standard solution contains 1 mgm of nitrogen (as ammonium sulphate) Nesslerized

lized in a 100 cc flask and the colorimeter prism of the standard is set at 20 millimeters $\frac{50}{R} \times D$ in which R stands for the reading of the unknown and D represents the volume to which its ammonia has been diluted gives the desired figure The reason for the figures is that we are here working with 0.4 cc of blood

When 5 cc of blood is taken and it is diluted to 50 the formula becomes $\frac{40}{R} \times D$

When working with human blood and taking 10 cc of the filtrate obtained from 5 cc of blood diluted to 50 the formula is $\frac{20}{R} \times D$

It may be thought that we are using unnecessarily small amounts of blood in these analyses We are, however, by no means sure that working with larger amounts would yield more accurate results and we have satisfied ourselves by scores of duplicate analyses that the method as outlined gives trustworthy figures Further, the smaller the quantity of blood which can be made to give reliable results the greater becomes the usefulness of the method The work which we have already done on cats could not have been done on such a small animal except by means of these microchemical methods Finally, small amounts of blood must be used for the urea determinations because of the disturbing effects of the sugar present (see p 520)

IV DETERMINATION OF UREA

Having described in some detail the preliminary treatment of the blood for the removal of the proteins and also the procedure for determining the total non-protein nitrogen, the urea determination in blood can be described very briefly

Five cubic centimeters of the alcoholic filtrate from cat's blood (or 10 cc from human blood) are taken for each determination This amount is measured into one of the large Jena test tubes in which the decomposition is to be made A drop of dilute acetic acid and two or three of kerosene are added and the test tube is then closed by a two-hole rubber stopper Through one of the holes in the stopper passes a glass tube drawn out to a capillary

several inches long The capillary end reaches nearly to the bottom of the test tube Through the other hole passes a short bent glass tube which is connected with a good water pump (see p 523) The test tube is placed in warm water and the vacuum pump is started In ten to thirty minutes the combined action of the gentle heat, the air current (through the capillary) and the vacuum removes all the alcohol The rubber stopper is then removed and the capillary tube is broken off by bending it against the sides of the test tube and is left there Two cubic centimeters of 25 per cent acetic acid, a temperature indicator, a pebble and 7 grams of dry potassium acetate are added and the decomposition of the urea is accomplished by heating it to 153 to 158°C for about eight to ten minutes exactly as in the urea determination described for urine (see p 515)

The ammonia set free by the subsequent air current treatment is collected in a large test tube, there Nesslerized (usually with only 3 cc of the diluted reagent), is made up to volume in a 10 cc volumetric flask and the color comparison is made as in the case of the total non-protein nitrogen against the same standard solution of ammonium sulphate We usually Nesslerize the total nitrogen, and the urea, and the standard, all at the same time Since only 10 cc is available of the solution corresponding to the urea, all of it must be poured into the Duboseq colorimeter cylinder for the making of the color comparison Dry cylinders must therefore be used If only one cylinder is available the urea should be read first We find it extremely convenient, however, to have several extra cylinders for the colorimeter and are thus able to read a series of urea determinations without stopping to rinse and wipe the inside of the cylinder for each determination

V DETERMINATION OF THE AMMONIA

The accurate determination of the ammonia in blood is beset with far greater difficulties than any of the earlier inventors of methods for its estimation have realized The blood decomposes spontaneously (and particularly in the presence of alkalies capable of setting free the ammonia) at all temperatures even when kept on ice The ammonia thus produced by decomposition in the course of a few hours is much greater than the preformed ammonia

present in the strictly fresh blood and when distillation methods are applied, whether in the vacuum or otherwise, the determination becomes little else than a measure of the decomposition

The decomposition in tissues such as the liver is even greater than in the blood and for this reason (among others) we are of the opinion that there is not a single experiment on record proving that macerated liver tissue splits off by hydrolysis the NH_2 groups from ordinary amino-acids when the latter are added to such tissue

In view of the instability of blood or of certain components of blood the determination of its ammonia can be accomplished with a reasonable degree of accuracy only by the speediest kind of a process. Having once thoroughly realized this fact the problem of determining this ammonia became with us a problem of learning to work with the smallest possible amount of material—a serious problem in view of the minute quantities of ammonia present in normal blood

The Nesslerization process lends itself as does none other to the quantitative estimation of small amounts of ammonia but instead of working with milligrams, as in urine, or with tenths of a milligram, as with blood in the estimation of total nitrogen and urea, it became a question of working with hundredths of a milligram. The quality of the color produced by Nessler's reagent with ammonium salt depends greatly on the amount of ammonia present, the tint is yellow or yellowish green when the amount of ammonia is very small (see p 496) and such faintly colored solutions can not be read in a Duboscq colorimeter as ordinarily used. It would of course have been possible to fall back on the procedure as it is used and has been used for a long time in water analysis, but we felt sure that this old process is not as reliable as the ammonia determinations we made by the help of a high grade colorimeter

By means of two important modifications of the Duboscq colorimeter we have succeeded in meeting all the necessary conditions

The chief reason why a dilute Nesslerized solution cannot be read against a much stronger one is that the light is absorbed in passing through a deep layer of the solution. Two such fields cannot therefore be made to look alike. After having unsuccessfully tried various kinds of screens for reducing the amount of

light passing through the thin layers of concentrated solutions we finally attained the desired result by the help of an iris diaphragm attached to one side of the colorimeter. By means of this diaphragm we are able to make use of 0.5 mgm of nitrogen as a standard and against it read a solution containing only a few hundredths of a milligram of ammonia nitrogen.

The second modification consists in the use of a 100 mm polariscope tube as container for our unknown ammonia solutions instead of the cylinders which go with the Duboscq colorimeter. These cylinders are so large in diameter that the solutions would have to be made impracticably dilute in order to furnish a reasonably high column. Ten cubic centimeters, for example, will reach to a height of only about 30 mm in the Duboscq cylinders yet these are about the smallest colorimeter cylinders in the market. With 10 cc we can, however, comfortably fill a 100 mm polariscope tube and, as it happens, such tubes just fit the Duboscq colorimeter when the solid movable glass prism is removed.

In the determination of the traces of ammonia here under discussion two precautions, not needed in any of the other methods described in the preceding three papers, are necessary. The first is that too much Nessler reagent must be avoided. The greenish tint observed in very dilute ammonia solutions when Nesslerized is almost wholly due to an excess of the reagent (see p. 497). The second precaution is the necessity of using only water that is strictly free from ammonia for diluting the unknown. The amount of ammonia in ordinary distilled water is sufficient to introduce a considerable error in this determination, while in those previously described it does not matter, partly because the ammonia is so small as to be negligible in view of the fact that the standard and the unknown are diluted to about the same extent with the same water. In this case where we read through 100 mm of the unknown solution against about 10 mm of the standard the case is different and the ammonia of the water must be eliminated.²

² Ammonia-free water is easily obtained from ordinary distilled water by the addition of a little saturated bromine water and a few drops of concentrated caustic soda. See *Claessen's Text-book*, II, p. 116. Such water containing hypobromite and alkali cannot of course be used for the absorption of the ammonia but only for diluting the reagent and for the final dilution to a definite volume.

We do not use such ammonia-free water for the small amount, 2 to 3 cc, employed for the absorption of the ammonia, but only for the water subsequently added in Nesslerizing and making up to a volume

The method for the determination of the ammonia is as follows

Ten cubic centimeters of systemic blood or 5 cc of portal or mesenteric blood are drawn in the usual manner (described above) by means of a pipette and transferred directly to one of the large Jena test tubes so extensively used in this work. To it are added 2 to 3 cc of the oxalate-carbonate solution described on p 524 (15 per cent potassium oxalate and 10 per cent sodium carbonate) and about 5 cc of toluol. The air current is then started and is run as fast as the apparatus can stand for 20 to 30 minutes. The liberated ammonia is collected, as previously described, in another large test tube charged with 5 to 6 drops of tenth-normal acid and 1 cc of water.

On account of the strong air current available in this laboratory, and also because of the relatively long period during which the process is carried out, we have found it desirable to cover the top of the test tube receiver with a small funnel from which the stem has been removed, thus obviating any loss which might be caused by spattering. At the end of the time indicated the contents of the receiver is Nesslerized in the usual manner but more cautiously, adding in all not over 1 cc of the previously diluted reagent (dilution 1:5). The solution is then carefully transferred to a 10 cc volumetric flask, diluted to the 10 cc mark, mixed, and with this solution the 100 mm polariscope tube is filled and closed as for ordinary polariscope work.

Two standard solutions, one containing 0.5 mgm the other 1 mgm of nitrogen, are Nesslerized simultaneously with the unknown solution made up to volume (100 cc) and one or the other is used as a standard.

In this case, of course, the unknown remains stationary and the standard solution must be adjusted until the two colors match.

In making this comparison it is necessary to keep moving both the diaphragm and the colorimeter prism in the standard solution until the right position of each is secured.

The colorimeter, as thus used, represents, we believe, a new departure in colorimetry and we are taking steps to secure the

making of such instruments. So far we have used an ordinary diaphragm taken from a microscope and have fastened it by means of two screw clamps on top of the colorimeter platform on which stands the cylinder. A new zero point has of course to be established to allow for the altered position of the cylinder. Now we are compelled to use one instrument exclusively for such ammonia determinations but we hope later to see such instruments properly made by some manufacturer.

In view of the fact that we have already published³ a number of ammonia determinations, made as described above, it seems unnecessary to insert more figures here. We do not assert that even those figures may not ultimately be found to be too high but we do believe that they represent the nearest approach to the true values that have yet been published.

We believe that the methods described in this paper will be found more serviceable than any hitherto available for the study of many important problems which can be solved only on the basis of blood and tissue analysis. We have so far published two papers (*loc cit*) and shall soon publish another more extensive one on the fate of the amino-acids absorbed from the digestive tract (and the gradual formation of urea). We hereby expressly revoke our earlier reservation (*loc cit*) of the field of research referred to in those papers by means of these methods. We would like to reserve for a while, however, the use of the methods for clinical investigations. We wish particularly to investigate nephritic cases and fevers, and for this purpose are now gathering data as to the variations in the composition of normal blood. The retention of 3 to 4 grams of non-protein nitrogen in a person of average size should be easily demonstrable by means of these methods unless the normal variations are greater than we have yet found them.

³ This *Journal*, xi, p 161, 1912

ON UROCANIC ACID

By ANDREW HUNTER

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In 1908 the writer isolated from a long-continued pancreatic digest of casein a crystalline substance, which he was able shortly afterwards to identify as urocanic acid ("Urocaninsäure") Up to that time the substance had been known only as an occasional constituent of the urine of dogs Two cases of its occurrence had been reported, the first in 1874 by its discoverer Jaffé,² and the second in 1898 by Siegfried.³ It would seem that the two animals concerned presented a rare anomaly of metabolism, not attributable to any definite cause Jaffé examined the urine of other dogs, and also of men, without again encountering the condition Siegfried could find no urocanic acid in 110 liters of human urine

The origin and constitution of urocanic acid have till now remained uncertain Siegfried conjectured a relation to the purines The appearance of the substance among the products of casein digestion pointed at once in another direction In preliminary communications⁴ I indicated the probability that the mother substance of urocanic acid is histidine Observations made soon afterwards suggested a more definite conclusion regarding its structure, but the hypothesis formed could not at the time be decisively tested without a fresh supply of material Efforts to procure this have consumed a great deal of time During the last three

¹ The substance discussed in the present communication was isolated at Edinburgh, and identified as urocanic acid at Leeds The remainder of the investigation was carried out at Cornell

Jaffé *Ber d chem Ges*, vii p 1668, 1874, and viii, p 811 1875

² Siegfried *Zeitschr f physiol Chem*, xxv, p 399, 1898

³ Hunter *Journ of Physiol*, xxxvii Proc Physiol Soc, p xxxvii 1908, and this *Journal*, vi, Proc Soc Biol Chem p xliii 1908-9

years large quantities of casein have been subjected for six or seven month periods to tryptic digestion, and in the product urocanic acid has been sought by the method which originally led to its isolation ⁵ Disappointment has been the uniform result, the original experience has not once been duplicated Attempts to discover a dog which excreted urocanic acid have met with no greater success The statement of Swain⁶ that a possibly related substance may occur in coyote urine led to an examination of that source also, but the animal investigated produced neither Swain's substance nor urocanic acid ⁷

It is therefore fortunate that recent work by others has afforded the means of deciding immediately the question at issue, and of settling the problem of constitution The circumstance removes any reason that may have hitherto existed for withholding the details now communicated

The digest from which urocanic acid was obtained had been made with the object of preparing a supply of the "polypeptide of pancreatic digestion" described by Fischer and Abderhalden ⁸

In 5 liters of water, containing 10 cc of concentrated *Liquor ammoniae*, there were dissolved 500 grams of "Plasmon" and 10 grams of "pankreatin absolut, Rhenania" The mixture was maintained at 40° in the presence of abundant toluene and chloroform Two days later 10 cc of ammonia, and sixteen days later 10 grams of pancreatin were added Digestion was continued for seven months, at the end of which time the biuret reaction was but feebly positive A loose jelly-like clot (plastein?), impregnated with tyrosine crystals, was filtered off, and the filtrate was concentrated *in vacuo* at 40° to 50° The second crop of tyrosine crystals having been removed, the liquid was diluted to about 6 liters, and treated with phosphotungstic acid The washed precipitate was decomposed in the cold with baryta, and excess of the latter removed by sulphuric acid The product was concentrated *in vacuo* and dried over sulphuric acid The yield of crude "polypeptide" was 56 grams This was dissolved in 1 liter of 5 per cent

⁵ In the somewhat laborious operations involved I had during the Summer Session of 1911 the assistance of Miss Ruth Wheeler, to whom I here gratefully acknowledge my indebtedness

⁶ Swain *Amer Journ of Physiol*, **viii**, p 30, 1905

⁷ Hunter and Givens *This Journal*, **viii**, p 449, 1910

⁸ Fischer and Abderhalden *Zeitschr f physiol Chem* **xxxix** p 81 1903

sulphuric acid, and the precipitation with phosphotungstic acid was repeated. From the precipitate were finally obtained 42 grams of brownish-yellow, extremely hygroscopic material, which dissolved in water with strongly alkaline reaction, and gave no biuret reaction whatever.

From this product it was decided to separate, if possible, arginine and histidine. One would naturally have expected the material to contain these bases in considerable amounts. It did give the intense "diazo reaction" shown by histidine. But it was found that towards silver nitrate and fixed alkali it did not react in the way expected of an arginine solution. As a matter of fact subsequent application of the silver-baryta method showed that neither arginine, nor yet histidine, was present in quantities that could be isolated. On the other hand there was produced by silver nitrate alone a quite considerable precipitate, which dissolved at once in the slightest excess of either acid or alkali. Attention was thereupon directed to the separation of the substance so reacting.

To this end the whole material was brought into aqueous solution (600 to 700 cc.), very nearly neutralized with nitric acid, and treated with 10 per cent silver nitrate as long as a precipitate resulted. Six or seven grams of the nitrate were required. The light brown gelatinous precipitate was collected on a filter and thoroughly washed. It was then suspended in water, and dissolved by the aid of a little dilute sulphuric acid. The solution was freed from silver by hydrogen sulphide, from the latter by a stream of air, and from sulphuric acid by baryta. It reacted now acid, and on concentration deposited 1.45 grams of crude crystalline material. This was purified by boiling with charcoal and by several crystallizations. The final yield was 0.92 gram. The amount originally present in the digest must have been considerably greater.

The substance thus obtained was sparingly soluble in cold, readily soluble in hot, water. Its solubility in alcohol was very slight, while in ether, acetone, ethyl acetate and carbon disulphide it was almost, if not quite, insoluble. It was dissolved with ease by glacial acetic acid, and by aqueous ammonia or sodium hydroxide. Its aqueous solution reacted acid to litmus. When rapidly crystallized from hot water, it formed branching groups of slender, beautifully iridescent, doubly refracting needles, sometimes nearly a centimeter long, on more gradual separation it appeared as well formed tetragonal prisms of the first and second orders.

The water-free substance melted with decomposition at 224° (corrected)

ANALYSIS AND MOLECULAR WEIGHT DETERMINATION 0.1369 gram air-dried substance lost 0.0281 gram at 110°

0.2208 gram, dried at 110° , yielded 0.4182 gram CO_2 and 0.0879 gram H_2O

0.1193 gram gave 21.1 cc N at 17° and 746 mm

0.1081 gram, dissolved in 10.48 grams glacial acetic acid, depressed the freezing point of the solvent 0.324°

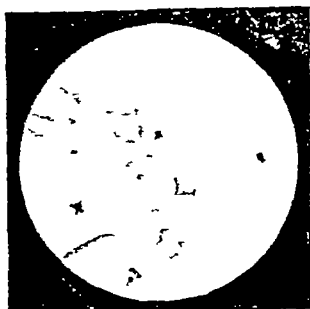
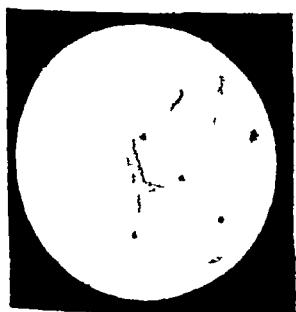
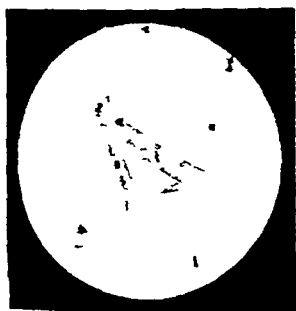
	Calculated for $\text{C}_6\text{H}_6\text{O}_2\text{N} \cdot 2\text{H}_2\text{O}$	Found
H_2O	20.7	20.5
C	52.1	51.7
H	4.4	4.5
N	20.3	20.5
Molecular weight	138.0	124.0

In crystalline form, solubility, melting point, and elementary composition the substance agreed exactly with the descriptions of urocanic acid. The two following reactions removed any uncertainty as to its identity therewith. (1) If a small quantity of the hydrated substance is treated with a drop of glacial acetic acid the crystals at first dissolve, but almost immediately thereafter, especially if the solution is shaken or rubbed, they separate again as a thick mass of small, opaque, white needles. This behavior is described by Siegfried as characteristic of urocanic acid. The opaque needles dissolve readily on addition of water or alcohol, or an excess of acetic acid. (2) When an aqueous solution of the substance is treated with an equal volume of 50 per cent nitric acid, a heavy microcrystalline precipitate of the nitrate is very rapidly deposited. The crystals of urocanic acid nitrate, produced in a similar manner, have a highly characteristic appearance. They are described by Jaffé as "small plates, bent in the form of a sickle, with the ends apparently frayed or eaten away, frequently several such plates are united to cross- or rosette-shaped aggregates." A reference to the photographs reproduced⁹ will demonstrate the quotation to be an exact description of the crystals yielded by the substance from casein.

Concerning the identity of the latter there was therefore no reasonable room for doubt. But the molecular weight determination above reported led to the formula $\text{C}_6\text{H}_6\text{O}_2\text{N}_2$. This was in

⁹ The negatives were very kindly made for me by Dr. R. Cattley, University of Leeds, whom I take this occasion of thanking for the service.

disagreement with the view of Jaffé (concurred in by Siegfried), which assigned to the acid the double formula $C_{12}H_{12}O_4N_4$ ¹⁰ Jaffé's choice was determined by the single circumstance, that urocanic acid, when heated, yields by loss of carbon dioxide and water a base—"urocanine"—to which apparently must be ascribed the formula $C_{11}H_{10}ON_4$. The evidence for the chemical individuality of this substance is not altogether convincing. Neither the base itself nor its salts with mineral acids could be obtained in crys-



VARIOUS FORMS OF UROCANIC ACID NITRATE

talline form. Its formula rests entirely upon analyses of an exceedingly hygroscopic chloroplatinate. The nature of urocanine

¹⁰ It was at first suspected that the determination itself might be in error. It was made with an apparatus, the only one then at my disposal which did not exclude moisture. Experiment proved that it was nevertheless easy to obtain with the same instrument and the same sample of acetic acid satisfactory approximations to the calculated molecular weight of other organic substances.

decidedly calls for further investigation. Whatever may be the mechanism of its formation, the sequel will show with sufficient clearness that urocanic acid at any rate does possess the simpler formula indicated by its cryoscopic effect.

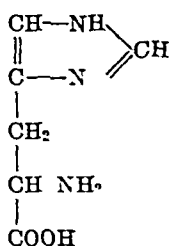
To the older descriptions of urocanic acid I am able to add the following points. Its solutions are optically inactive. They are precipitated by silver nitrate, the precipitate increases in bulk upon exact neutralization with ammonia, but dissolves instantly in the slightest excess of either ammonia or nitric acid. Urocanic acid is precipitated also by mercuric chloride, and by phosphotungstic and picrolonic acids. The *phosphotungstate* dissolves in hot water, from which it crystallizes in minute cubes or short rectangular prisms. The *picrolonate*, gelatinous when formed by bringing together aqueous solutions but granular if precipitated in alcohol, is dissolved with great difficulty in boiling absolute alcohol, with less difficulty in boiling water, and with comparative ease in boiling dilute alcohol. It can be recrystallized from water as bright yellow sheaves of long filamentous needles, from 75 per cent alcohol as dense clumps of yellow plates, which singly take the form of elongated rhombs. It decomposes about 268° (corrected), after gradual discoloration from about 230° .

On bringing together saturated aqueous solutions of urocanic acid and picric acid there is no immediate precipitate, but there gradually separate yellow iridescent macroscopic prisms of the *picrate*, which melt at 224° to 225° (corrected).

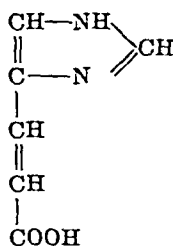
Urocanic acid in dilute sodium carbonate solution gives a very intense red reaction with diazobenzenesulphonic acid. It does not evolve any nitrogen on treatment with nitrous acid. It instantly reduces a cold alkaline permanganate solution with immediate liberation of manganese dioxide.

A substance giving the diazo reaction, and obtained from protein in the way described, could hardly be other than an imidazole derivative, standing in some relation to histidine. Its precipitation reactions were in harmony with this conclusion. The immediate reduction of cold alkaline permanganate, which the imidazole ring itself will not bring about, pointed to the possession of an unsaturated side chain. These considerations, taken in conjunction with the empirical formula $C_6H_6O_2N_2$, suggested the probability that urocanic acid is an imidazole-acrylic acid, related

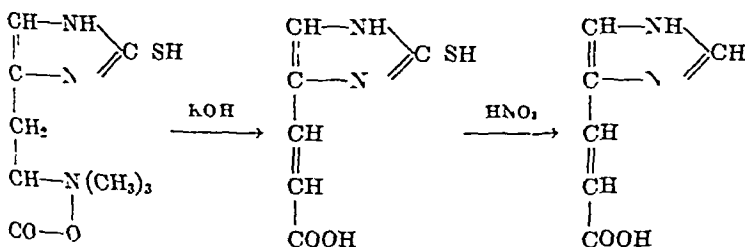
therefore to histidine in the same way as cinnamic acid is to phenylalanine¹¹



Histidine

Imidazole-acrylic
or Urocanic acid

Such a constitution would account for the acid reaction to indicators with simultaneous possession of basic characters, the want of optical activity, and the failure to react with nitrous acid. But before it could be held to be fully established, confirmatory evidence of a stricter nature was essential. This can now be supplied. A substance known independently to have the structure represented above has been recently described by Barger and Ewins¹². They obtained it in two ways (1) from ergothioneine (the betaine of thiohistidine) in the manner illustrated by the scheme below



¹¹ This view of the constitution of urocanic acid occurred independently, as I learn from a private communication, to Professor Treat B. Johnson of Yale University, whose studies on thiohydantoins have led him to an interest in the derivatives of histidine (see Johnson and Guest *Amer. Chem. Journ.*, xlvii, p. 242, 1912). It was Professor Johnson who drew my attention to the paper of Barger and Ewins mentioned below and he therefore who furnished the stimulus that occasioned the immediate publication of my results. It is a pleasure to record my appreciation of the friendly spirit that has characterized Professor Johnson's side of the correspondence.

¹² Barger and Ewins *Journ. Chem. Soc.* xcix, p. 2336, 1911.

(2) by the action of trimethylamine on α -chloro- β -imidazole-propionic acid. Of this product sufficient has been placed in my possession¹³ to enable me to say with certainty that it is identical with urocanic acid. It crystallizes in precisely the same forms, it behaves in the same way with glacial acetic acid, and its nitrate has the peculiar and characteristic shape of urocanic acid nitrate. Specimens of the two products heated side by side melted together at 231° to 232° (corrected),¹⁴ a mixture of both in equal proportions melted simultaneously with a sample of the compound from casein. Barger and Ewins describe a phosphotungstate crystallizing in small rectangular plates, and a picrate forming golden yellow prisms.

The comparison places it beyond reasonable doubt that urocanic acid is β -imidazole-4(or 5)-acrylic acid.

That such a substance should make its appearance in a pancreatic digest is somewhat astonishing. Its origin certainly cannot be ascribed to the action of trypsin. What the responsible factor was, whether the particular ferment preparation employed contained a deaminizing enzyme of peculiar nature, or whether the responsibility lay with some accidental circumstance in the manipulation of the product, it has not been possible to determine. It is doubtless of significance that from the digestion mixture arginine, as well as histidine, had disappeared. The attempt to duplicate the occurrence has not been abandoned, and an explanation may yet be found. One naturally thinks of bacterial action. The incubated mixture at no time exhibited evidence of organismal growth, yet in the absence of bacteriological control that source of decomposition cannot be by any means excluded. With this in mind I have grown some of the commoner organisms in casein and histidine solutions. The result hoped for has not so far been attained. Experiments in this direction also are being continued, although, so far as I am aware, no analogous case of the conversion by bacteria of an amino- into an unsaturated acid has been reported.

¹³ To Drs. George Barger and Arthur J. Ewins I take this opportunity of expressing my grateful recognition of the courtesy with which they at once acceded to my request for a specimen.

¹⁴ I had previously found for urocanic acid the melting point 224°, while Barger and Ewins report for their substance 235° to 236°. The fact is, as Siegfried also noticed, that the value found varies widely with the manner of heating. This is probably equally true for the picrate, which according to Barger and Ewins melts at 213° to 214°, according to my own determination at 224° to 225°.

The appearance of imidazole-acrylic acid under the circumstances described in this paper is not more remarkable than its occasional occurrence as an excretory product in the dog. In this character it almost certainly represents an intermediate step in the catabolism of histidine. The type of amino-acid transformation which would thus be illustrated apparently occurs in plants—witness the formation of cinnamic and *p*-cumaric acids—but has not hitherto been met in animals. The production of cinnamoyl-glycocoll observed by Dakin¹⁵ to follow administration of phenylpropionic acid to cats presents perhaps the nearest analogy. Other origins than the one assumed are of course not impossible. But the formation of the unsaturated acid is not the only problem offered. The phenomenon of its excretion is equally puzzling. It is known that moderate doses of cinnamic acid are easily and completely oxidized within the animal organism.¹⁶ A case where the analogous imidazole derivative cannot be similarly disposed of is almost certainly a metabolic anomaly. The elucidation of the structure of urocanic acid adds therefore a fresh interest to the search, still being prosecuted, for an animal which regularly excretes that substance. It would be of interest to determine whether even normal dogs do not excrete small quantities of urocanic acid in response to enteral or parenteral administration of histidine or its derivatives. Experiments to decide the point are in contemplation. The metabolic fate of histidine has been the subject of studies by Abderhalden and Einbeck,¹⁷ Abderhalden, Einbeck and Schmid,¹⁸ Kowalevsky,¹⁹ and Dakin²⁰. But in none of the experiments reported was urocanic acid specifically sought.

¹⁵ Dakin. *This Journal* v, pp 173 and 303, 1908, also vi, p 203, 1909.

¹⁶ Cohn. *Zeitschr f physiol Chem*, xvii, p 274, 1893, and Dakin. *this Journal* v, p 413, 1909.

¹⁷ Abderhalden and Einbeck. *Zeitschr f physiol Chem*, lxi, p 322, 1909.

¹⁸ Abderhalden, Einbeck, and Schmid. *Ibid*, lxxiii, p 395, 1910.

¹⁹ Kowalevsky. *Biochem Zeitschr*, xliii, p 1, 1910.

²⁰ Dakin. *This Journal*, v p 499, 1912.

ON SPHINGOSINE¹

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Sphingosine was discovered by Thudichum² on hydrolysis of a cerebroside, phrenosine. Discussing the chemical properties of the substance, its behavior towards bases and acids, the author took into consideration the possibility of the substance having the structure of an amino-acid or of an alkaloidal base. In his final conclusion he expressed preference to the view of the basic nature of the substance.

In later years Thierfelder³ repeated the work of Thudichum, and in the main substantiated his views. The work of Thierfelder, however, was directed principally to the study of the properties of

¹ A report on the results of the present investigation has appeared in the Proceedings of the Meeting of the American Society of Biological Chemists, held December 28th to December 30th, 1911, published in the March number of this Journal. In Heft 6, vol lxxvii of Hoppe-Seyler's *Zeitschrift für physiologische Chemie*, published on April 9, there appeared two articles by Thierfelder, Riesser and Thomas in which the authors arrived at the same conclusions as reported by us. The appearance of the two articles was caused undoubtedly by the publication of our report, since Heft 5 of vol lxxvii of Hoppe-Seyler's *Zeitschrift* contained no mention of Thierfelder's name among the authors of twenty-six articles received for publication. In a footnote to one of the articles Professor Thierfelder claims the sole privilege for work on sphingosine and related substances, for the reason that Thudichum's work had been half forgotten at the time when Thierfelder directed his attention to cerebroside. We do not feel that this justifies the request made by Thierfelder, that the work which had been in progress in our laboratory for more than a year should be abandoned before it is completed.

² *Die chemische Konstitution des Gehirns*, Tübingen, 1901.

³ *Zeitschr f physiol Chem*, xlv, p 366, 1905, Kitagawa *Ibid*, xlix, p 236, 1906.

the cerebroside, which he named "cerebron" The author expressed no definite view regarding the chemical structure of the base

The results of the present investigation have made certain that sphingosine is an unsaturated monoaminodihydroxyalcohol

This conclusion is based on the following data

1 The substance contains all its nitrogen in form of primary amino nitrogen

2 The presence of a double binding in the molecule is demonstrated by the readiness with which sphingosine absorbs hydrogen when treated according to the method of Paal A substance is thus formed which has the composition of dihydrosphingosine It was analyzed in the form of a sulphate and a triacetyl derivative

3 The presence of two hydroxyl groups in the molecule is evident from the fact that sphingosine forms a triacetyl derivative which no longer contains the original primary amino group The substance forms a dimethylether, and finally it can be reduced to an amine, sphingamine

As yet it is not certain whether or not the carbon atoms are linked in a normal chain Attempts were made to reduce the dihydrosphingosine to the corresponding amine, but instead of the heptadecylamine there was always obtained the unsaturated sphingamine Efforts to obtain the saturated amine are now in progress Also work is in progress on the respective position of the double bond, and of the hydroxyl groups

EXPERIMENTAL PART

Sphingosine

The base was obtained on hydrolysis of "cerebrin" prepared by a slight modification of the process described by Parcus⁴ The conditions of hydrolysis were similar, but not identical with those described by Thierfelder The base was prepared in crude form as the sulphate, which was then transformed into the free base and into the acetate The discoverer of the base mentioned that it could be made to crystallize out of ether It was found in course

⁴ *Journ f prakt Chem*, *xiv*, p 310, 1881

of this work that crystallization proceeded much more readily out of petroleic ether

The sulphate was obtained in the form of a white crystalline powder. It melted with decomposition at 233° to 234°C (uncorrected). A great many samples were analyzed. The analysis of one of these gave the following results

0.0996 gram of the substance dried in chloroform-vacuum bath over phosphorus pentoxide gave on combustion 0.0940 gram of H_2O and 0.2280 gram of CO

0.2400 gram of the substance, employed for a Kjeldahl nitrogen estimation, required for neutralization 6.9 cc of $\frac{\text{N}}{16}$ acid

	Calculated for $(\text{C}_{17}\text{H}_{23}\text{NO}_2)_2\text{H}_2\text{SO}_4$	Found
C	61.08	61.05
H	10.78	10.60
N	4.19	4.06

The optical activity of the substance was the following

0.5304 gram of the sulphate was dissolved in a mixture of 5 cc of chloroform and 1 cc of glacial acetic acid. The total weight of solution was 8.7514 grams. The rotation in pure D-light was -1.50° , hence

$$[\alpha]_D^{20} = -13.12^{\circ} (\pm 0.00)$$

Diacetate Dissolved in glacial acetic acid and petroleic ether, the substance crystallized in form of very long needles of the following composition

0.1402 gram of the substance gave on combustion 0.1290 gram H_2O and 0.3206 gram of CO_2

	Calculated for $\text{C}_{17}\text{H}_{23}\text{NO}_2 \cdot (\text{C}_2\text{H}_3\text{O}_2)_2$	Found
C	62.22	62.36
H	10.61	10.22

Amino nitrogen estimation A solution of 0.300 gram of the sulphate in 10.0 cc of glacial acetic acid was employed for an amino nitrogen estimation according to the method of Van Slyke. Five cc were used for each experiment. All nitrogen was given off in thirty minutes. In each experiment 11 cc of nitrogen were formed at $t = 21^{\circ}\text{C}$ and $p = 760$ mm

	Calculated for $(\text{C}_{17}\text{H}_{23}\text{NO}_2 \cdot \text{NH}_2)_2\text{H}_2\text{SO}_4$	Found
	4.19	4.17

Dihydrosphingosine

The hydrogen absorption value of sphingosine is obtained most conveniently when the free base is dissolved in ether and shaken with aqueous colloidal palladium prepared according to Paal, 0.100 gram of palladium to about 0.500 gram of the base dissolved in about 150 cc of absolutely pure ether gave the most satisfactory results. The absorption was completed in about thirty minutes. The velocity of the operation was greatly increased by the addition of 1 cc of glacial acetic acid to the ethereal solution.

0.500 gram of the substance absorbed 50 cc of hydrogen (without correction for t and p). Theory requires 45 cc of H.

0.6486 gram of the substance absorbed 59 cc of H, theory requires 56 cc

The ethereal solution of dihydrosphingosine was evaporated to dryness and the substance converted into the sulphate and into the triacetyl derivative.

The sulphate was obtained in form of a white crystalline powder. Its melting point was only slightly different from the unsaturated compound, being 235°C.

	Calculated for (C ₁₇ H ₃₇ NO ₂) ₂ H ₂ SO ₄	Found
C	60.61	60.90
H	11.38	11.11

The optical activity of the substance was difficult to determine for the lack of a sufficiently satisfactory solvent. Approximately it was as follows:

0.0776 gram of the substance dissolved in about 3 cc of alcohol containing sulphuric acid, and weighing 2.8640 grams gave a rotation of -0.29° in a 2 dm tube.

$$[\alpha]_D^{20} = -10.67^\circ$$

0.1214 gram of the substance gave on combustion 0.1153 gram of H₂O and 0.2948 gram of CO₂.

	Calculated for C ₁₇ H ₃₁ NO ₂ (CH ₃ CO) ₃	Found
C	66.76	66.76
H	10.50	10.60

Acetyl derivatives

On treatment of the free base with acetic anhydride di- or triacetylsphingosine can be obtained. The first is obtained by dissolving the base in boiling acetic anhydride and evaporating the solution under diminished pressure. The triacetyl derivative is prepared by allowing the base to digest with acetic anhydride in a boiling water bath with return condenser for one hour and only then evaporating the solution to dryness. The further treatment in both instances is identical. The residue obtained on evaporating the solution under diminished pressure is taken up in chloroform and again evaporated under diminished pressure. This residue is taken up in hot acetone and the substance allowed to crystallize. For analysis the substances were dried in a vacuum-chloroform bath over phosphorus pentoxide.

Diacetyl derivative 0.1317 gram of the substance gave on combustion 0.1256 gram of H_2O and 0.3283 gram of CO_2 .

0.2720 gram of the substance was dissolved in 10 cc of glacial acetic acid and used for amino nitrogen estimation according to Van Slyke. Five cubic centimeters of the solution were employed for each experiment. There was formed 9.3 cc nitrogen at $24^\circ C$ and 758 mm pressure. The substance was allowed to react one hour, although the reaction was practically completed in twenty minutes.

	Calculated for $(C_{17}H_{27}O_2 \cdot NH_2) (CH_3CO)_2$	Found
C	68.1	68.16
H	10.8	10.59
N	3.4	3.78

The physical constants and saponification value of this substance were not determined.

Triacetyl derivative 0.1194 gram of the substance gave on combustion 0.1064 gram of H_2O and 0.2950 gram of CO_2 .

0.2500 gram of the substance was dissolved in 10 cc of glacial acetic acid and employed for an amino nitrogen estimation. No formation of nitrogen took place.

	Calculated for $C_{17}H_{27}NO_2 \cdot (CH_3CO)_3$	Found
C	67.15	67.38
H	9.98	9.98

The substance melted sharply at 102° to $103^\circ C$ (uncorr.)

0.3383 gram of the substance was dissolved in 60 cc of methyl alcohol, containing 10 cc of a $\frac{1}{2}$ solution of sodium hydrate in methyl alcohol. The solution was heated on boiling water bath for two hours, allowed to stand over night and titrated. It required 24.90 cc of $\frac{1}{16}$ alkali to neutralize the acetic acid formed on saponification. The theory required 24.75 cc.

Dimethylsphingosine

This substance is formed in course of hydrolysis of cerebrosides by means of methylalcohol and mineral acid. Thierfelder, who was the first to have the substance in his hands, erroneously regarded it as a new base. Since our first communication, Thierfelder and Riesser⁵ substantiated our view on the substance. The methyl derivative was obtained in form of a sulphate on concentrating the mother liquors from the crude sphingosine sulphate. The sulphate was then transformed into the free base and this again transformed into the hydrochloride. The hydrochloride crystallizes out of alcohol in the form of large glittering plates.

The substance was identified by the fact that, similarly to sphingosine, it contained all its nitrogen in form of primary amino nitrogen, it contained one unsaturated bond and on boiling with hydroiodic acid formed the required amount of methyl iodide.

0.1615 gram of the substance gave on combustion 0.1654 gram H_2O and 0.3926 gram of CO_2 .

	Calculated for $C_{19}H_{33}NO \cdot HCl$	Found
C	65.18	66.65
H	11.52	11.24

Hydrogen absorption value. One gram of the substance dissolved in ether containing 2 cc of glacial acetic acid. On treatment with palladium according to Paal it absorbed 67 cc of hydrogen. Theory requires 72.6 cc.

Amino nitrogen estimation. 0.3500 gram of the hydrochloride dissolved in 10 cc of glacial acetic acid. Five cc of this solution used for amino nitrogen estimation according to Van Slyke. There formed 12.5 cc of nitrogen at $t = 24^\circ$ and $p = 758$ mm.

	Calculated for $C_{19}H_{33}O_2 \cdot NH_2 \cdot HCl$	Found
N	4.01	4.00

Methyl estimation. 0.1206 gram of the hydrochloride boiled with hydroiodic acid of specific gravity = 1.71 in the apparatus of Zeisel and Fanto. There was obtained 0.1286 gram of silver iodide.

	Calculated for $C_{17}H_{25}NO_2 \cdot (CH_3)_2 \cdot HCl$	Found
CH_3	8.45	7.00

The physical properties of the substance did not permit a more accurate estimation. In a control experiment with sphingosine

⁵ *Zeitschr f physiol Chem*, LVIII, p. 508, 1912.

crystallized out of petroleic ether no silver iodide was formed. On the other hand, the base obtained directly after removing the sulphuric acid from apparently pure sphingosine sulphate still caused the formation of some silver iodide. The highest value obtained in this manner was equivalent to $\text{CH}_3 = 2.32$ per cent.

Sphingamine

Attempts were made to reduce dihydrosphingosine to the corresponding amine. The normal heptyldecylamine has been obtained synthetically. Hence a comparison of the two bodies should have determined the fact whether or not the substances were identical. In several experiments the reduction was attempted by means of hydroiodic acid, and in one experiment the dihydrosphingosine was transformed into the dihydrodichlorsphingosine, which was then reduced by means of metallic sodium and alcohol.

However, under all conditions the unsaturated substance was formed.

Reduction with hydroiodic acid was carried out in sealed tubes at 125°C . The reaction-product was dissolved in ether. The ethereal solution was dried with anhydrous sodium sulphate, and then diluted with one-third of its volume of 98 per cent alcohol, and the solution treated with metallic sodium.

The substance obtained from the solution was transformed into the sulphate. The analysis of the substance obtained from three different experiments follows.

- I 0.1042 gram of the substance gave 0.1100 gram H_2O and 0.2562 gram of CO
- II 0.1014 gram of the substance gave 0.0998 gram H_2O and 0.2494 gram of CO
- III 0.1153 gram of the substance gave 0.1233 gram H_2O and 0.2800 gram of CO_2

0.1480 gram of sample I was used for Kjeldahl nitrogen estimation. It required for neutralization 4.1 cc. of $\frac{N}{10}$ acid.

	Calculated for ($\text{C}_{17}\text{H}_{35}\text{N}\cdot\text{H}_2\text{SO}_4$)	Found		
		I	II	III
C	67.46	67.2	67.2	67.85
H	12.02	11.82	11.05	11.97
N	4.52	4.44	4.51	

Reduction of dihydriodichlorsphingosine The chlorderivative was obtained by digesting dihydrosphingosine with thionylchloride in a water bath at 50°. The crude substance without purification was dissolved in a mixture consisting of two parts of ether and one of 98 per cent alcohol and reduced with metallic sodium. The substance obtained in this manner was transformed into the sulphate and analyzed.

0.1251 gram of the substance gave on combustion 0.1310 gram of H₂O and 0.3097 gram of CO₂.

	Calculated for (C ₁₇ H ₃₃ N) H SO ₄	Found
C	67.46	67.51
H	12.02	11.72

ERRATUM

On page 217 of this volume, No. 3, tenth line from the top, for *strict* read *generic*.

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